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Rouleau, J. K.

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Dissertation

THE COLOR REACTIONS OF ANTIMONY CHLORIDES WITH SOME NATURAL DERIVATIVES OF PHENANTHRENE

BY

John Kiernan Rouleau

(S.B., Massachusetts Institute of Technology, 1928;
S.M., Massachusetts Institute of Technology, 1932.)

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Doctor of Philosophy,
1937

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A C K N O W L E D G E M E N T

I should like to take this opportunity to thank Burnham Sarle Walker, Ph.D.,M.D., of the Boston University School of Medicine under whose direction this investigation was carried out. I should also like to thank Rev. A. B. Langguth, S.J., of the Department of Chemistry of Boston College for his help and cooperation, David C. O'Donnell Ph.D., also of the Department of Chemistry of Boston College and Albert J. Plummer, Ph.D., of the Boston University School of Medicine for their help and timely suggestions. Doctor Gregory Pincus of Harvard University is to be thanked for his contributions of oestriol and oestradiol.

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I N T R O D U C T I O N

The ability of antimony chlorides to form a complex compound with organic substances has been studied by many investigators. Some of the compounds, indicated by an asterisk (*), form colored complexes, and are the basis of certain color tests, e.g. Carr & Price[6] tests and Steinle & Kahlenberg[40] tests. A chronological list of some investigators who employed antimony trichloride in this manner follows:

Smith[38] in 1879 announced reactions of antimony trichloride with naphthalene, anthracene, phenanthrene*, diphenyl, 3-isomeric-dinaphthyls, stilbene*, triphenyl methane*, chrysene*, pyrene*, conin, nicotine, morphine*, codein*, narcotin*, thebaine*, papaverin, narcein*, strychnine, brucine*, chinin, cinchonin, veratrin*, atropine, aconitin*, santonin*.

Smith & Davis[39] in 1882 published their findings on the reactions of the trichloride with naphthalene and benzene to yield a complex compound.

Rosenheim & Stelman[30] in 1901 determined the composition of the complex formed with benzene and toluene.

May[22] in 1911 determined the composition of the complex formed with aniline, o-toluidine, p-toluidine, and p-chloraniline.

Vanstone[43] in 1911 determined the complexes formed with s-diphenyl ethane, azobenzene, stilbene, and

benzyl.

May[22] in 1912 discussed the complexes formed with a series of diazonium compounds.

Kahlenberg[19] in 1922 discussed the reactions of the trichloride with the sterols and certain terpenes.

Vanstone[43] in 1925 reported the complexes with benzanilide* and benzylanilide*.

Carr & Price[6] studied the color complexes of the trichlorides and oils containing vitamin A, in 1925.

Willimot, Moore & Wokes[47] in 1926 studied the reaction of the trichloride in reference to its use as a test for vitamin A.

Wokes[50] studied the color reactions of the trichloride with the sterols and cholesterol derivatives. He also studied the effect of the pentachloride on the same compounds, in 1928.

Heilbron[17] working with the sterol group from 1929 to 1936 has described color reactions of the trichloride with derived sterols and sterol hydrocarbons.

Goldhammer[12] in 1929 studied the reactions of the trichloride on the sterols and female sex hormones.

The more important workers who studied the complexes formed with the pentachloride are listed below.

Williams[46] in 1876 reported the formation of complexes with methyl alcohol*, ethyl alcohol, and ethyl

ether.

Zetter[51] in 1878 reported the action of the pentachloride on phenanthrene.

Diehl[10] in 1878 reported the action of the pentachloride on anthracene*, anthraquinone*, and alizarin*.

Merz and Weith[24] in 1883 studied the action of the pentachloride on phenanthraquinone, diphenyl glycolic acid*, diphenylene acetic acid, benzidine*, carbazol*, triphenyl and diphenylene phenyl methane*, rosaniline*, dibenzyl, ditoyl*, pyrene*, chrysene*, diphenyl*, triphenyl benzol*, benzonitrile, alpha and beta naphthonitrile.

Rosenheim & Stelman[30] in 1901 reported the action of the pentachloride on pyridine*, chinolin, dimethyl aniline, acetaldehyde*, benzaldehyde, acetone, ethyl benzoate, benzoyl chloride, acetamide, phthalic anhydride, succinic acid, oxalic acid, nitrobenzene*, benzol and toluol.

Rosenheim & Lowenstein[29] in 1902 reported the action of the pentachloride on benzoic acid, phenyl acetic acid*, dimethyl oxalate*, malonic acid*, ethyl carbonate*, and lactic acid*.

Rosenheim & Levy[28] in 1904 reported the action of the pentachloride on ethyl cinnamate*, cinnamic acid*, cinnamylidenacetone*, cinnamic aldehyde, dibenzyl ace-

tone*, cinnamylidenbenzylacetone*.

Rosenheim, Weinland, & Schmid[31] in 1905 reported the action of the pentachloride on pyridine*.

Meyer[25] in 1908 reported the action of the pentachloride on alpha naphtha quinone*, benzophenone*, and anthraquinone*.

Hilpert & Wolff[18] in 1913 reported the action of the pentachloride on benzol*, thiophene*, diphenyl*, naphthalene*, anthracene*, carbazol*, phenanthraquinone*, phenanthrene*, di and tri phenyl methane*, fluoren*, toluol, ortho, meta, and para xylene*, mesitylene*, cymene*, and triphenyl chlormethane*.

Thomsen[41] in 1911 reported the action of the pentachloride on quinine*, quinidine*, cinchonine*, chinconidine*, morphine*, codein*, nicotine*, cocaine*, caffeine*, and strychnine*.

Steinle & Kahlenberg[40] in 1926 reported the action of the pentachloride on cholesterol*, phytosterols*, glycocholic acid, taurocholic acid, lecithin*, cephalin*, and certain terpenes.

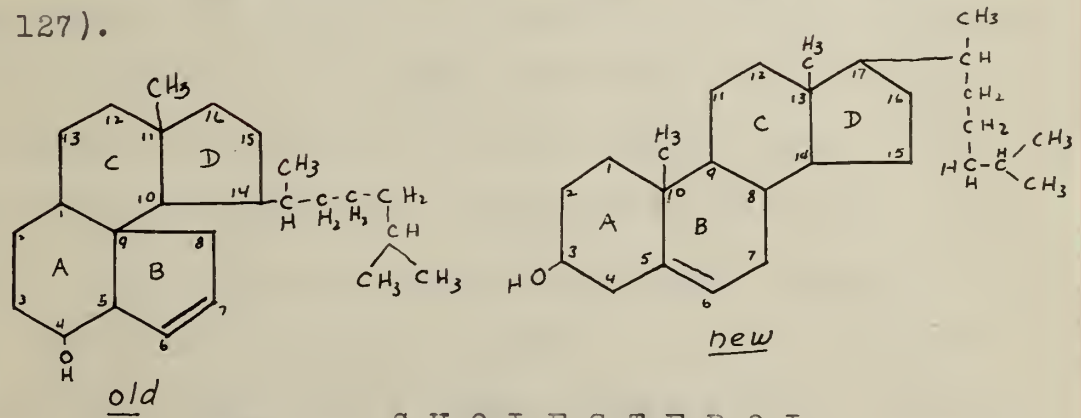
Goldhammer[12] in 1929 reported the use of the pentachloride as a precipitating agent in obtaining the sex hormones.

The results of Carr&Price and Steinle & Kahlenberge

have been modified to give colorimetric methods for the determination of vitamin A and cholesterol respectively.

A review of the literature indicated that there were many cases of contradictory findings. Thus Steinle & Kahlenberg reported that using the trichloride no color developed with cholesterol. On the other hand Wokes using the Carr & Price reagent (the trichloride in $CHCl_3$) reported a red color resulting with cholesterol. Montignie[25] reported a violet color with cholesterol. In preliminary tests by the author using the pentachloride reagent ergosterol was found to be much more reactive than cholesterol and yielded red colors. Steinle & Kahlenberg found that the phytosterols required longer periods of time to develop a blue or violet color. Steinle & Kahlenberg found that they could detect 0.000125 gms. of cholesterol while the author using a different strength pentachloride reagent could detect 0.0000654 gms. of cholesterol (See Series B, Effect of concentration of cholesterol). In conducting their tests Wokes, and Steinle & Kahlenberg purified their cholesterol by fractional distillation and obtained a product with melting points from 147.9 - 148.5°C. It has been pointed out by Anderson[1] that pure cholesterol can only be obtained through the dibrom compound and has a melting point of 150 - 151°C.

Moreover, the work of these investigators occurred before 1932 in which year the structure of the sterols was proven to be a perhydro 1-2, cyclo penteno phenanthrene ring system by Weiland & Dane and Rosenheim & King (Fieser[11] page 127).



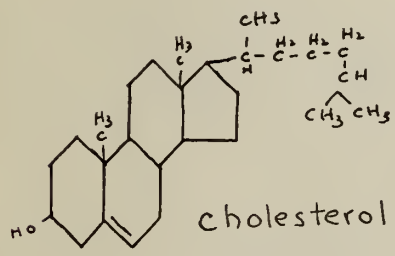
C H O L E S T E R O L

Since the bile acids, sterols, sex hormones, and vitamin D_2 are included in this ring system it was felt that a re-investigation of the color reactions with the antimony chlorides would be worth while. Particularly since it is only within the last few years that their structural interralationships have been proven. Moreover, the color reactions are quite sensitive so that the question as to purity of the compounds tested was believed to be the probable explanation for the differences in results previously obtained. In the tests carried out by the author the purity of the compounds tested is believed to be of such a high order that this possibility is reduced to a minimum.

To avoid a possible misinterpretation of the term Vitamin D_2 an explanatory note seems essential. This is a synonomous term with Calciferol. The term was originated by Windaus in 1931. Fieser[11] in his work uses the term exclusively. In 1930 when crystalline vitamin D was originally obtained it was named calciferol by the English investigators and vitamin D_1 by the Windaus group. Later it was determined that this crystallized product was a mixture. The English investigators retained the name Calciferol for the pure active compound - while the Windaus group named their active isomer vitamine D_2 to differentiate it from the original mixture. In this paper the nomenclature will follow that employed by Fieser.

A further point of minor importance is the method of recording the color reactions. The author having carried out some preliminary tests wondered whether the color was, for example, "reddish-orange", "pinkish", or "orange", as the colors are variously recorded. Hence, for his own benefit, at least, an attempt was made to classify the colors as much as possible according to the standards set down in Mulliken's "Treatise on the Identification of Pure Organic Compounds". In many substances it was impossible to find a suitable standard and when such cases occurred recourse was made to the unsatisfactory system of naming the color. The compounds investigated were cholesterol, ergosterol,

oestriol, oestradiol, oestrone, testosterone acetate, cholic acid, apocholic acid, dehydrocholic acid, desoxycholic acid, glycocholic acid, phenanthrene, and vitamine D_2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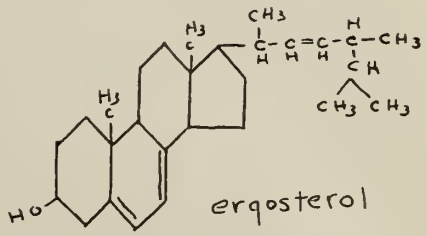
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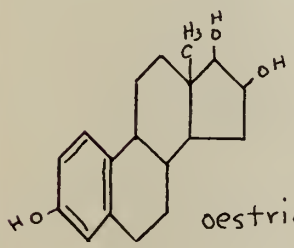
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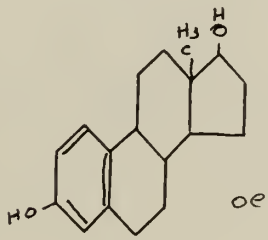
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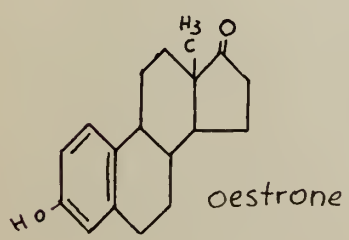
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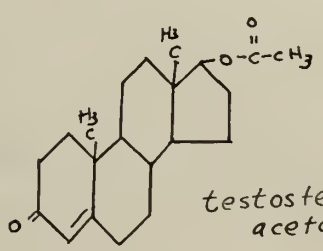
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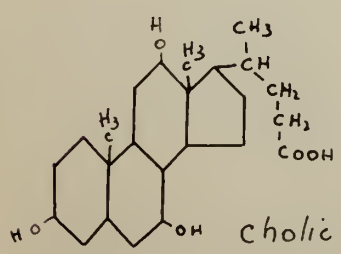
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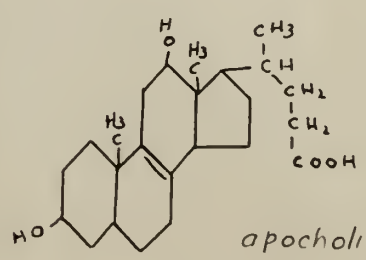
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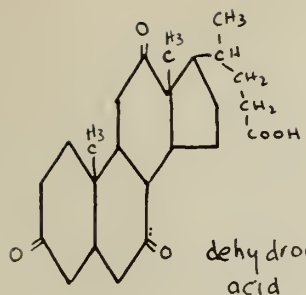
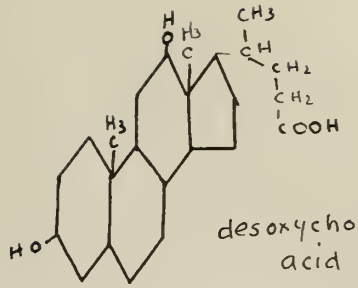
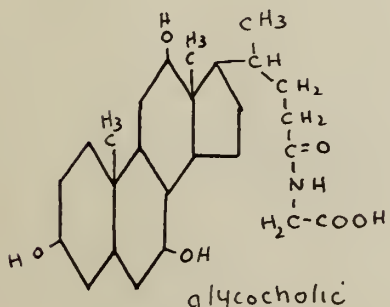


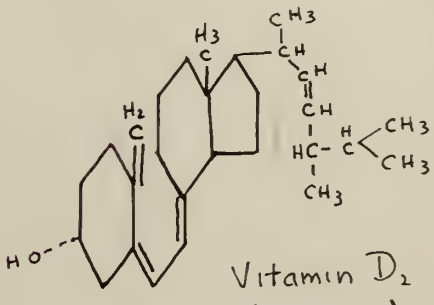
dehydrocholic acid



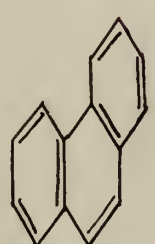
desoxycholic acid



glycocholic acid



Vitamin D_2 (Calciferol)



phenanthrene

EXPERIMENTAL

MATERIALS USED

CHOLESTEROL

A commercial sample of cholesterol labeled "C.P." was found to have a melting point 134-140°C.

This product was purified by the method of Windaus and Hauth[49] through the dibromo compound with subsequent reduction in alcoholic solution with zinc dust and acetic acid. Although Fieser states that the method of Schoenheimer[37] using sodium iodide in absolute alcohol is more suitable than the above method it was used since there is no possibility of the formation of pinacones which is the salient feature with the Schoenheimer method and the added step of preparing absolute alcohol could be avoided. The product obtained is very satisfactory and had a melting point of 150.6°C. Anderson[1] gives the melting point as 150-151°C.

For this melting point and all others Anschütz thermometers and a Thiele tube were used.

ERGOSTEROL

Samples of specially purified ergosterol were kindly sent to the author through the courtesy of the Fleishmann Laboratories. The melting point of the product was 160°C. It had an optical rotation $(\alpha)_D^{27^\circ}$ equals -132°. The recorded specific rotation is -133°. The recorded melting point is 160-162°C. Callow[5] points out that the optical rotation is a much better criterion of purity.

BILE ACIDS

These products were obtained from Reidel-de-Haen. In reviewing the literature for solubility and melting point standards it was found that Fieser for example gives the melting point of desoxycholic acid as 176.0°C, while Heilbron[16] lists three separate melting points for this compound depending upon the method of purification. These were 144-145°C from glacial acetic acid; 153-155°C from alcohol and ether; 172-173°C from acetone and for the anhydrous acid. This apparent discrepancy is due to the fact that the bile acids in general have the property of forming very stable complexes and crystallize from solution with a molecule of solvent so firmly bound that it can be removed only by heating under reduced pressure for a very long time. Desoxycholic acid forms a molecular compound with stearic acid that melts rather sharply at 186°C and the amount of fatty acid present is so small that it is hardly apparent from the analytical figures. (Fieser page 130). Weiland & Sorge[44] studying this phenomenon noted that many water-insoluble compounds could be brought into aqueous solution by means of the formation of molecular compounds with desoxycholic acid giving rise to the "Choleic acids". Accordingly on checking the melting points of the compounds studied belonging to this group a sharp melting point was taken as an indication of purity

even though the melting point obtained could not be found in the literature. For example, the melting point of the glycocholic acid was found to be 142°C. Reference to Heilbron gave the melting point of glycocholic acid as from 132-152°C depending upon the method of preparation.

Compound	M.P. found	M.P. recorded	Reference
Apocholic Acid	167.1°C	170-171°C	3
Cholic Acid	199.8°C	195, 198°C	16, 4
Dehydrocholic Acid	233.3°C	231-232°C	27
Desoxycholic Acid	171-172°C	172-173°C	16
Glycocholic Acid	142°C	132-152°C	16

It was impossible to obtain pure taurocholic acid commercially and the isolation of the compound from the bile is unsatisfactory (Fieser page 125). The only satisfactory way to obtain this compound is by direct synthesis from taurine and cholic acid according to the method of Cortese and Baumann[8]. Since it is also a saturated bile acid not readily undergoing dehydrogenation this synthesis was not carried out.

Phenanthrene

Ten grams of phenanthrene were purchases from the Eastman Kodak Co. and repurified through the picrate as outlined in Fieser. Melting point 99°C. Recorded 100°C

Reference standard, Beilstein[2].

OESTRONE

Ten mgs. of pure oestrone were obtained through the courtesy of Parke-Davis. The product had been prepared through the semi-carbazone and had a melting point of 260°. Recorded melting point is 259°C. Reference, Fieser[11].

TESTOSTERONE ACETATE

A sample of testosterone acetate was obtained through the courtesy of the Ciba Co. The product is a synthetic material obtained by the method of Ruzicka & Wettstein[34].

VITAMIN D_2

One-tenth gram of crystalline vitamin D_2 was donated by the Winthrop Chemical Co.

ANTIMONY TRICHLORIDE

Baker's C.P. Analyzed $SbCl_3$ was used. The melting point was found to be 71°C. Recorded 72-73.4°C. Reference Mellor[23].

ANTIMONY PENTACHLORIDE

Baker's C.P. analyzed $SbCl_5$ was used. The melting point was found to be +0.5°C. Recorded, -6° to 3.5°C. Reference, Mellor[23].

CHLOROFORM

This product was C.P. $CHCl_3$ redistilled from calcium chloride and had a boiling point 61.2 - 61.8°C.

OESTRIOL

A few milligrams of this compound, which had been chemically and biologically standardized, were obtained through the courtesy of Dr. Gregory Pincus of Harvard University.

OESTRADIOL

A small sample of this material, which had been chemically and biologically standardized, was also obtained through the thoughtfulness of Dr. Gregory Pincus.

PREPARATION OF TEST SOLUTIONS

APOCHOLIC ACID

40 mgs. made up to 100 cc. in 95% alcohol.

CHOLESTEROL

130.8 mgs. made up to 100 cc. in 95% alcohol.

CHOLIC ACID

1.0253 grams made up to 100 cc. in 95% alcohol.

DEHYDROCHOLIC ACID

29.9 mgs. made up to 100 cc. in 95% alcohol.

DESOXYCHOLIC ACID

24.9 mgs. made up to 100 cc. in 95% alcohol.

GLYCOCHOLIC ACID

59.6 mgs. made up to 100 cc. in 95% alcohol.

OESTRONE

10 mgs. made up to 100 cc. in 95% alcohol.

OESTRADIOL

2.1 mgs. made up to 50 cc. in 95% alcohol.

OESTRIOL

1.1 mgs. made up to 50 cc. in 95% alcohol.

PHENANTHRENE

24.8 mgs. made up to 100 cc. in 95% alcohol.

TESTOSTERONE ACETATE

37.9 mgs. made up to 50 cc. in $CHCl_3$.

ERGOSTEROL (1)

30.2 mgs. made up to 100 cc. in 95% alcohol.

ERGOSTEROL (2)

11.4 mgs. made up to 50 cc. in $CHCl_3$.

VITAMIN D_2

23.9 mgs. made up to 100 cc. in $CHCl_3$.

$SbCl_3$ in $CHCl_3$

Saturated solution in chloroform.

$SbCl_3$ in C_2H_5OH

10 grams of the trichloride made up to 100 cc. in 95% alcohol to which had been added enough concentrated HCl to remove turbidity.

$SbCl_3$ in GLACIAL ACETIC ACID

10.6 grams made up to 100 cc. in glacial acetic acid.

$SbCl_5$ in $CHCl_3$

16.2 grams made up to 100 cc. in $CHCl_3$.

$SbCl_5$ in C_2H_5OH

10 grams made up to 100 cc. in 95% alcohol to which had been added enough concentrated HCl to remove turbidity.

$SbCl_5$ in GLACIAL ACETIC ACID

26.7 grams made up to 100 cc. in glacial acetic acid.

ROSENHEIM REAGENT

9 grams of C.P. trichloracetic acid in 1 gram of water.

$SbCl_3$ in CONCENTRATED HCl (HORMONE REAGENT)

50% by weight $SbCl_3$ in concentrated HCl.

METHOD OF TESTING

The sample to be tested is pipetted into a clean dry 4" test tube and carefully evaporated just to dryness on a water bath. The tube is allowed to cool and then the antimony reagent added. Any color developed is recorded. If the sample is to be heated this is accomplished on a water bath. After heating the color of the resultant material is again noted. Finally when the tube is cool the sample is diluted and the color recorded.

For tests in which the sample is not heated after addition of the antimony reagent the sample is diluted directly. The color of the diluted sample in either case is recorded.

With each sample the following experiments were carried out:

(1) Using a constant amount of sample the quantity of antimony reagent is varied.

(2) Using a constant amount of the antimony reagent the quantity of test substance was varied.

(3) Using the optimum conditions as determined from the two preceding runs the sample was heated on a water bath for varying periods of time.

SPECIAL TEST FOR OESTRIOL

The sample to be tested is pipetted into a dry 4" test tube and carefully evaporated just to dryness on a water bath. When cool 0.1 cc. of concentrated H_2SO_4 is added. The tube is then heated for two to three minutes on a water bath, and diluted with 0.5 cc. of water. 3 drops of the

of the acid antimony trichloride reagent (HORMONE REAGENT) are added and the tube reheated on a water bath for seven minutes. A blue color results.

SPECIAL TESTS FOR OESTRONE AND OESTRADIOL

The sample to be tested is pipetted into a dry 4" test tube and carefully evaporated just to dryness on a water bath. When cool 0.05 cc. of the hormone reagent is added and the tube heated on the water bath for thirty minutes. After cooling 0.5 cc. of water is added followed by seven drops of concentrated HCl to suppress hydrolysis. A red color results.

D A T A

SERIES A

ERGOSTEROL

Solution of Ergosterol in 95% alcohol. 1 cc. contains 0.302 mgs.

Preliminary Tests

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.5 $SbCl_5$	2 minutes	OR Tint #2	GB Tint #2
2	0.1	0.5 $SbCl_5$	0 minutes	OR Tint #2	Colorless
3	0.1	0.5 $SbCl_5$	0 minutes	YO normal	RO Tint #1
4	0.1	0.5 $SbCl_5$	2 minutes	YO normal	OY Tint #2

ALL SAMPLES DILUTED WITH 2 cc. $CHCl_3$

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.1 $SbCl_5$	2 minutes	Y broken tone	Blue
2	0.1	0.3 $SbCl_5$	2 minutes	YO Shade 1	Blue
3	0.1	0.5 $SbCl_5$	2 minutes	O Shade 1	GB Tint #2
4	0.1	0.8 $SbCl_5$	2 minutes	O Shade 2	Colorless

SERIES A (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
5	0.1	1.0 $SbCl_3$	2 minutes	O Shade 1	Colorless
6	0.1	1.5 $SbCl_3$	2 minutes	RO normal	Pink (turbid)
7	0.1	2.0 $SbCl_3$	2 minutes	RO normal	Pink (turbid)
8	0.1	0.05 $SbCl_3$	2 minutes	RO Shade 1	Blue
		ALL SAMPLES DILUTED WITH 2 cc. $CHCl_3$			
1	0.1	0.1 $SbCl_5$	0 minutes	RO normal	GB Tint #2
2	0.1	0.3 $SbCl_5$	0 minutes	YO normal	OR Tint #2
3	0.1	0.5 $SbCl_5$	0 minutes	YO normal	R Tint #2
4	0.1	0.8 $SbCl_5$	0 minutes	YO normal	YO Tint #1
5	0.1	1.0 $SbCl_5$	0 minutes	YO normal	OY Tint #1
6	0.1	1.5 $SbCl_5$	0 minutes	OY normal	OY normal
7	0.1	2.0 $SbCl_5$	0 minutes	OY normal	OY normal
8	0.1	0.05 $SbCl_5$	0 minutes	O Shade 1	B Tint #2
		ALL SAMPLES DILUTED WITH 2 cc. $CHCl_3$			

SERIES A (Continued)

Effect of concentration of Ergosterol

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.5 $SbCl_3$	2 minutes	YO Tint #1	Colorless
2	0.05	0.5 $SbCl_3$	2 minutes	O Tint #1	Colorless
3	0.10	0.5 $SbCl_3$	2 minutes	OR Tint #2	GB Tint #2
4	0.15	0.5 $SbCl_3$	2 minutes	OR Tint #2	GB Tint #2
5	0.20	0.5 $SbCl_3$	2 minutes	OR Tint #2	GB Tint #2
6	0.50	0.5 $SbCl_3$	2 minutes	VR Tint #1	BG Broken Tone
7	1.0	0.5 $SbCl_3$	2 minutes	BG Shade 2	B Broken Tone

Sample No. 7 became slightly turbid. When warmed for about a minute solution became clear and color changed to BV Tint #1.

All Samples Diluted with 2 cc. $CHCl_3$

1	0.01	0.5 $SbCl_5$	0 minutes	Y Tint #2	Colorless
2	0.05	0.5 $SbCl_5$	0 minutes	Y normal	O Tint #2
3	0.10	0.5 $SbCl_5$	0 minutes	YO normal	RO Tint #2
4	0.15	0.5 $SbCl_5$	0 minutes	YO Shade 1	OR Tint #1

SERIES A (Continued)

Effect of concentration of Ergosterol

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
5	0.20	0.5 $SbCl_5$	0 minutes	0 Shade 1	OR Tint #1
6	0.50	0.5 $SbCl_5$	0 minutes	0 Shade 1	R Tint #1
7	1.00	0.5 $SbCl_5$	0 minutes	Sherry wine shade	VR normal

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.20	0.5 $SbCl_3$	0 minutes	OR Tint #2	Colorless
2	0.20	0.5 $SbCl_3$	1 minute	0 Shade 1	BG Tint #2
3	0.20	0.5 $SbCl_3$	4 minutes	0 Shade 2	BG Tint #2
4	0.20	0.5 $SbCl_3$	9 minutes	0 Shade 2	RV Tint #2
5	0.20	0.5 $SbCl_3$	19 minutes	0 Shade 2	YO Tint #2
6	0.20	0.5 $SbCl_3$	39 minutes	---------	O Tint #2

In this run Tubes No. 5 & 6 showed a strong fluorescence. On standing overnight the following changes were noted: (a) Tube No. 1-GB Tint #2, (b) Tubes No. 2 & 3 remained the same, (c) Tube No. 4-V Broken tone, (d) Tube No. 5-RV Tint #2, (e) Tube No. 6-RV Tint #2.

SERIES A (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.5 $SbCl_5$	0 minutes	OY normal	RO Tint #2
2	0.1	0.5 $SbCl_5$	1 minute	OR Shade 1	YO Tint #1
3	0.1	0.5 $SbCl_5$	4 minutes	OY Shade 2	OY Tint #1
4	0.1	0.5 $SbCl_5$	9 minutes	OY Shade 2	OY Tint #2
5	0.1	0.5 $SbCl_5$	19 minutes	OY Shade 2	OY Tint #2
6	0.1	0.5 $SbCl_5$	39 minutes	OY Shade 2	Turbid

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES B

CHOLESTEROL

Solution of Cholesterol in 95% alcohol. 1 cc. contains 1.308 mgs.

Preliminary Tests

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	o.1	0.5 $SbCl_3$	0 minutes	Colorless	Colorless
2	o.1	0.5 $SbCl_3$	2 minutes	Y normal	RV Tint #2
3	o.1	0.5 $SbCl_5$	0 minutes	O Shade 2	YO Shade 2
4	o.1	0.5 $SbCl_5$	2 minutes	O Shade 2	YO Shade 1

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.05 $SbCl_3$	2 minutes	Colorless	Colorless
2	0.1	0.10 $SbCl_3$	2 minutes	OY normal	Colorless
3	0.1	0.3 $SbCl_3$	2 minutes	OY normal	RV Tint #2 slowly
4	0.1	0.5 $SbCl_3$	2 minutes	OY normal	RV Tint #2
5	0.1	1.0 $SbCl_3$	2 minutes	OY normal	Colorless

SERIES B (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
6	0.1	1.5 $SbCl_3$	2 minutes	OY normal	OY Tint #2
7	0.1	2.0 $SbCl_3$	2 minutes	OY normal	OY Tint #2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.05 $SbCl_5$	0 minutes	Muddy red brown	B Broken tone
2	0.1	0.1 $SbCl_5$	0 minutes	RO Shade 1	GY Broken tone
3	0.1	0.3 $SbCl_5$	0 minutes	O Shade 1	YO Shade 1
4	0.1	0.5 $SbCl_5$	0 minutes	O Shade 1	YO Shade 1
5	0.1	1.0 $SbCl_5$	0 minutes	YO Shade 1	YO Shade 1
6	0.1	1.5 $SbCl_5$	0 minutes	YO Shade 1	YO Shade 1
7	0.1	2.0 $SbCl_5$	0 minutes	YO Shade 1	YO Shade 1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of concentration of cholesterol

1	0.01	0.5 $SbCl_3$	2 minutes	Y Tint #1	Colorless
2	0.05	0.5 $SbCl_3$	2 minutes	Y Tint #1	Colorless

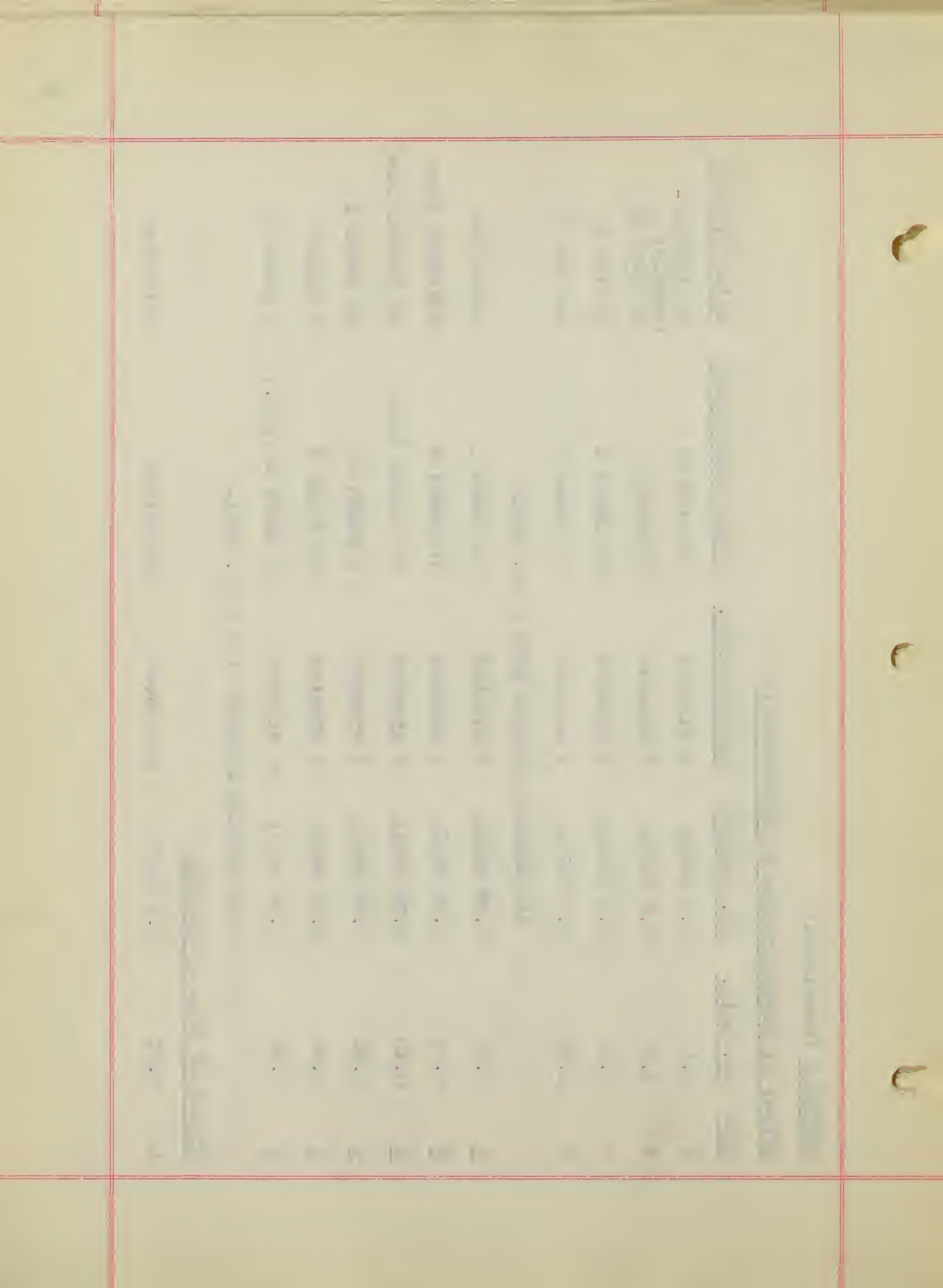
SERIES B (Continued)

Effect of concentration of cholesterol

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
3	0.1	0.5 $SbCl_3$	2 minutes	OY Tint #1	V Tint #2 - (slowly)
4	0.2	0.5 $SbCl_3$	2 minutes	O normal	OR Tint #2
5	0.5	0.5 $SbCl_3$	2 minutes	YO Shade 1	O Tint #1
6	1.00	0.5 $SbCl_3$	2 minutes	YO Shade 1	RO Tint #1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.01	0.05 $SbCl_5$	0 minutes	YO Shade 1	Colorless
2	0.05	0.05 $SbCl_5$	0 minutes	YO Shade 2	B Broken tone
3	0.10	0.05 $SbCl_5$	0 minutes	YO Broken tone	GY Broken tone
4	0.20	0.05 $SbCl_5$	0 minutes	O Shade 2	GB Shade 1
5	0.50	0.05 $SbCl_5$	0 minutes	RO Shade 2	B Shade 1
6	1.00	0.05 $SbCl_5$	0 minutes	O Shade 2 (ppt.)	B Shade 2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of time of heating

1	0.20	0.5 $SbCl_3$	0 minutes	Colorless	Colorless



SERIES B (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
2	0.20	0.5 $SbCl_3$	1 minute	Faint Pink	Colorless
3	0.20	0.5 $SbCl_3$	4 minutes	R Tint #1	OR Tint #2
4	0.20	0.5 $SbCl_3$	9 minutes	Y Broken tone	Y Broken tone
5	0.20	0.5 $SbCl_3$	19 minutes	Y Broken tone	Y Broken tone
6	0.20	0.5 $SbCl_3$	39 minutes	Y Broken tone	Turbid
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.20	0.05 $SbCl_5$	0 minutes	Dirty-black-brown	BG Shade 2
2	0.20	0.05 $SbCl_5$	1 minute	Dirty-black-brown	OY Shade 2
3	0.20	0.05 $SbCl_5$	4 minutes	Dirty-black-brown	OY Shade 2
4	0.20	0.05 $SbCl_5$	7 minutes	Dirty-black-brown	OY Shade 2
5	0.20	0.05 $SbCl_5$	10 minutes	Dirty-black-brown	OY Shade 2
6	0.20	0.05 $SbCl_5$	20 minutes	Dirty-black-brown	OY Shade 2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

SERIES C

O E S T R O N E

Solution of Oestrone in 95% alcohol. One cc. contains 0.1 mgs.

Preliminary Tests

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.05	0.5 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.05	0.5 $SbCl_3$	5 minutes	O Tint 1 (fluorescent)	OY Tint #2
3	0.05	0.5 $SbCl_5$	0 minutes	OY Shade 1	Colorless
4	0.05	0.5 $SbCl_5$	5 minutes	OY Shade 1	Colorless

On standing over night sample no. 2 became OR Tint #2.

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagant	heating mins.	color (undiluted)	color diluted
1	0.05	0.05 $SbCl_3$	5 minutes	OY normal	OR Tint #2
2	0.05	0.10 $SbCl_3$	5 minutes	OY normal	RO Tint #2
3	0.05	0.30 $SbCl_3$	5 minutes	YO Tint #1	O Tint #2

SERIES C (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
4	0.05	0.50 $SbCl_5$	5 minutes	YO Tint #1	O Tint #2
5	0.05	1.00 $SbCl_5$	5 minutes	YO Tint #1	Turbid
6	0.05	1.50 $SbCl_5$	5 minutes	YO Tint #1	Turbid
		ALL SAMPLES FLUORESCE			
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.05	0.05 $SbCl_5$	5 minutes	OY Shade 1	Colorless
2	0.05	0.10 $SbCl_5$	5 minutes	OY Shade 1	Colorless
3	0.05	0.30 $SbCl_5$	5 minutes	OY Shade 1	Colorless
4	0.05	0.50 $SbCl_5$	5 minutes	OY Shade 1	Colorless
5	0.05	1.00 $SbCl_5$	5 minutes	OY Shade 1	Colorless
6	0.05	1.50 $SbCl_5$	5 minutes	OY normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
2ND RUN WITH $SbCl_5$					
1	0.05	0.01 $SbCl_5$	5 minutes	OY normal	Colorless

SERIES C (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
2	0.05	0.02 $SbCl_3$	5 minutes	OY normal	OR Tint #2
3	0.05	0.03 $SbCl_3$	5 minutes	OY normal	OR Tint #2
4	0.05	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
5	0.05	0.05 $SbCl_3$	5 minutes	OY normal	OR Tint #2

ALL SAMPLES FLUORESCE.

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of concentration of Oestrone

1	0.01	0.04 $SbCl_3$	5 minutes	OY normal	Colorless
2	0.03	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
3	0.05	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
4	0.08	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
5	0.10	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
6	0.30	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2
7	0.50	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2

SERIES C (Continued)

Effect of concentration of Oestrone

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
8	1.00	0.04 $SbCl_3$	5 minutes	OY normal	OR Tint #2

TUBES 3 to 8 INCLUSIVE SHOW SAME COLOR BUT INCREASING INTENSITY AS AMOUNT OF OESTRONE IS INCREASED.

ALL SAMPLES FLUORESCE.

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of time of heating

1	0.05	0.04 $SbCl_3$	1 minute	OY Shade 1	Colorless
2	0.05	0.04 $SbCl_3$	5 minutes	OY Shade 1	OR Tint #2
3	0.05	0.04 $SbCl_3$	10 minutes	OY Shade 1	OR Tint #2
4	0.05	0.04 $SbCl_3$	20 minutes	YO normal	OR Tint #2
5	0.05	0.04 $SbCl_3$	30 minutes	O normal	OR Tint #2
6	0.05	0.04 $SbCl_3$	60 minutes	RO normal	OR Tint #2

TUBES 2 to 6 INCLUSIVE FLUORESCE

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES D

PHENANTHRENE

Solution of Phenanthrene in 95% alcohol. Each cc. contains 0.248 mgs.

Preliminary Tests

Tube	cc. soln.	cc. reagent	Heating mins.	color (undiluted)	color diluted
1	0.05	0.5 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.05	0.5 $SbCl_3$	2 minutes	Colorless	Colorless
3	0.05	0.5 $SbCl_5$	0 minutes	Colorless	Colorless
4	0.05	0.5 $SbCl_5$	2 minutes	OY Shade 2	Colorless

ALL SAMPLES DILUTED WITH 2 CC. CHLOROFORM

Effect of concentration of antimony reagent

1	0.05	0.1 $SbCl_3$	6 minutes	Colorless	Colorless
2	0.05	0.3 $SbCl_3$	6 minutes	Colorless	Colorless
3	0.05	1.0 $SbCl_3$	6 minutes	Colorless	Colorless
4	0.05	1.5 $SbCl_3$	6 minutes	Colorless	Colorless
5	0.05	2.0 $SbCl_3$	6 minutes	Colorless	Colorless

ALL SAMPLES DILUTED WITH 2 CC. CHLOROFORM

SERIES D (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.05	0.05 $SbCl_5$	3 minutes	YO Shade 1	Colorless
2	0.05	0.10 $SbCl_5$	3 minutes	YO Shade 1	Colorless
3	0.05	0.30 $SbCl_5$	3 minutes	YO Shade 1	Colorless
4	0.05	1.0 $SbCl_5$	3 minutes	YO normal	Y Shade 2
5	0.05	1.5 $SbCl_5$	3 minutes	OY Shade 2	GY Tint #2
6	0.05	2.0 $SbCl_5$	3 minutes	OY Shade 2	GY Tint #1

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of concentration of Phenanthrene

1	0.1	0.3 $SbCl_3$	2 minutes	Colorless	Colorless
2	0.5	0.3 $SbCl_3$	2 minutes	Colorless	Colorless
3	0.8	0.3 $SbCl_3$	2 minutes	Colorless	Colorless
4	1.0	0.3 $SbCl_3$	2 minutes	Colorless	Colorless
5	1.5	0.3 $SbCl_3$	2 minutes	Colorless	Colorless
6	2.0	0.3 $SbCl_3$	2 minutes	Colorless	Colorless

SERIES D (Continued)

Effect of concentration of Phenanthrene

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.3 $SbCl_5$	2 minutes	OY Shade 2	Colorless
2	0.5	0.3 $SbCl_5$	2 minutes	O Shade 2	Y Tint #2
3	0.8	0.3 $SbCl_5$	2 minutes	O Shade 2	OY normal
4	1.0	0.3 $SbCl_5$	2 minutes	O Shade 2	OY Shade 1
5	1.5	0.3 $SbCl_5$	2 minutes	O Shade 2	OY normal
6	2.0	0.3 $SbCl_5$	2 minutes	O Shade 2	YO Shade 1

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of time of heating

1	1.0	0.5 $SbCl_3$	1 minute	Colorless	Colorless
2	1.0	0.5 $SbCl_3$	3 minutes	Colorless	Colorless
3	1.0	0.5 $SbCl_3$	10 minutes	Colorless	Colorless
4	1.0	0.5 $SbCl_3$	15 minutes	Colorless	Colorless
5	1.0	0.5 $SbCl_3$	20 minutes	Colorless	Colorless

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES D (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	1.0	0.5 $SbCl_5$	1 minute	0 Shade 2	0Y Shade 1
2	1.0	0.5 $SbCl_5$	3 minutes	0 Shade 2	0Y Shade 1
3	1.0	0.5 $SbCl_5$	10 minutes	0 Shade 2	0Y Shade 1
4	1.0	0.5 $SbCl_5$	15 minutes	0 Shade 2	0Y Shade 1
5	1.0	0.5 $SbCl_5$	20 minutes	0 Shade 2	0Y Shade 1 (Turbid)

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES E

DEHYDROCHOLIC ACID

Solution of Dehydrocholic Acid in 95% alcohol. Each cc. contains 0.299 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.05 $SbCl_3$	5 minutes	Colorless	Colorless
2	0.1	0.12 $SbCl_3$	5 minutes	Colorless	Colorless
3	0.1	0.30 $SbCl_3$	5 minutes	OY Shade 1	Colorless
4	0.1	0.50 $SbCl_3$	5 minutes	OY Shade 1	Colorless
5	0.1	0.80 $SbCl_3$	5 minutes	OY Shade 1	Colorless
6	0.1	1.00 $SbCl_3$	5 minutes	OY Tint #1	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.05 $SbCl_5$	5 minutes	OY Shade 1	Colorless
2	0.1	0.10 $SbCl_5$	5 minutes	OY Shade 1	Colorless
3	0.1	0.30 $SbCl_5$	5 minutes	OY Shade 1	Colorless
4	0.1	0.50 $SbCl_5$	5 minutes	OY Shade 1	Colorless
5	0.1	0.80	5 minutes	OY Shade 1	Colorless

SERIES E (Continued)

Effect of concentration of Dehydrocholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.3 $SbCl_3$	3 minutes	Colorless	Colorless
2	0.3	0.3 $SbCl_3$	3 minutes	GY Tint #2	Colorless
3	0.5	0.3 $SbCl_3$	3 minutes	GY Tint #1	Colorless
4	1.0	0.3 $SbCl_3$	3 minutes	GY Tint #1	Colorless
5	2.0	0.3 $SbCl_3$	3 minutes	Y normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.3 $SbCl_5$	3 minutes	OY normal	Colorless
2	0.3	0.3 $SbCl_5$	3 minutes	OY normal	Colorless
3	0.5	0.3 $SbCl_5$	3 minutes	YO Shade 1	Y Tint #2
4	1.0	0.3 $SbCl_5$	3 minutes	Dark brown	Y Tint #2
5	2.0	0.3 $SbCl_5$	3 minutes	Dark brown	OY normal
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of time of heating

1	1.5	0.3 $SbCl_3$	0 minutes	Colorless	Colorless

SERIES E (Continued)

Effect of time of heating

Tube	Cc. soln.	cc. Reagent	heating mins.	color (undiluted)	color diluted
2	1.5	0.3 $SbCl_3$	3 minutes	Y normal	Colorless
3	1.5	0.3 $SbCl_3$	5 minutes	Y normal	Colorless
4	1.5	0.3 $SbCl_3$	10 minutes	Y normal	Colorless
5	1.5	0.3 $SbCl_3$	20 minutes	Y normal	Colorless
		ALL SAMPLED DILUTED WITH 2 CC. $CHCl_3$			
1	1.5	0.3 $SbCl_5$	0 minutes	Colorless	Colorless
2	1.5	0.3 $SbCl_5$	3 minutes	YO Shade 2	Y Tint #1
3	1.5	0.3 $SbCl_5$	5 minutes	YO Shade 2	OY normal
4	1.5	0.3 $SbCl_5$	10 minutes	YO Shade 2	OY normal
5	1.5	0.3 $SbCl_5$	20 minutes	YO Shade 2	OY Shade 1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

SERIES F

D E S O X Y C H O L I C A C I D

Solution of Desoxycholic Acid in 95% alcohol. Each cc. contains 0.249 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.05 $SbCl_3$	3 minutes	Y normal	Colorless
2	0.1	0.10 $SbCl_3$	3 minutes	Y normal	Colorless
3	0.1	0.15 $SbCl_3$	3 minutes	Y normal	Colorless
4	0.1	0.20 $SbCl_3$	3 minutes	Y normal	Colorless
5	0.1	0.3 $SbCl_3$	3 minutes	Y normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.05 $SbCl_5$	3 minutes	YO Shade 2	Colorless
2	0.1	0.10 $SbCl_5$	3 minutes	YO Shade 2	Colorless
3	0.1	0.15 $SbCl_5$	3 minutes	YO Shade 2	Colorless
4	0.1	0.20 $SbCl_5$	3 minutes	YO Shade 2	Colorless
5	0.1	0.30 $SbCl_5$	3 minutes	YO Shade 2	OY Tint #2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

SERIES F (Continued)

Effect of concentration of Desoxycholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.2 $SbCl_3$	3 minutes	Y Tint #2	Colorless
2	0.05	0.2 $SbCl_3$	3 minutes	Y Tint #2	Colorless
3	0.10	0.2 $SbCl_3$	3 minutes	Y Tint #1	Colorless
4	0.20	0.2 $SbCl_3$	3 minutes	Y Normal	Colorless
5	0.40	0.2 $SbCl_3$	3 minutes	Y Normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.01	0.2 $SbCl_5$	3 minutes	OY Shade 2	Colorless
2	0.05	0.2 $SbCl_5$	3 minutes	OY Shade 2	Colorless
3	0.10	0.2 $SbCl_5$	3 minutes	YO Shade 2	Colorless
4	0.20	0.2 $SbCl_5$	3 minutes	YO Shade 2	OY Tint #1
5	0.40	0.2 $SbCl_5$	3 minutes	YO Shade 2	OY Tint #1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of time of heating

1	0.10	0.2 $SbCl_3$	0 minutes	Colorless	Colorless

SERIES F (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
2	0.1	0.2 $SbCl_3$	3 minutes	GY normal	Colorless
3	0.1	0.2 $SbCl_3$	6 minutes	Y normal	Colorless
4	0.1	0.2 $SbCl_3$	9 minutes	Y normal	Colorless
5	0.1	0.2 $SbCl_3$	12 minutes	Y Shade 1	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.2 $SbCl_5$	0 minutes	OY Tint #1	Colorless
2	0.1	0.2 $SbCl_5$	3 minutes	YO Shade 2	Colorless
3	0.1	0.2 $SbCl_5$	6 minutes	Colorless	Colorless
4	0.1	0.2 $SbCl_5$	9 minutes	Colorless	Colorless
5	0.1	0.2 $SbCl_5$	12 minutes	Colorless	Colorless

Tubes 3, 4, and 5 contained white crystals which were insoluble in the chloroform.

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES G-1

A P O C H O L I C A C I D

Solution of Apocholic Acid in 95% alcohol. Each cc. contains 0.400 mgs.

Preliminary Tests

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.5	0.5 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.5	0.5 $SbCl_3$	2 minutes	Y normal	Turbid
3	0.5	0.5 $SbCl_5$	0 minutes	RO normal	O normal
4	0.5	0.5 $SbCl_5$	2 minutes	YO Shade 2	YO Shade 1

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of concentration of antimony reagent

1	0.5	0.05 $SbCl_3$	3.5 minutes	OY Shade 1	Colorless
2	0.5	0.10 $SbCl_3$	3.5 minutes	OY normal	Red ppt.*
3	0.5	0.30 $SbCl_3$	3.5 minutes	OY Shade 1	Y Shade 2
4	0.5	0.80 $SbCl_3$	3.5 minutes	Y normal	Y Shade 2
5	0.5	1.00 $SbCl_3$	3.5 minutes	Y normal	Y Shade 2

* Red ppt. (OR Shade 2) formed insoluble in $CHCl_3$.

SERIES G-1 (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
6	0.5	1.5 $SbCl_3$	3.5 minutes	Y normal	Y Shade 2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.5	0.05 $SbCl_5$	0 minutes	YO Shade 1	G Tint #1
2	0.5	0.10 $SbCl_5$	0 minutes	RO normal	Y Shade 2
3	0.5	0.30 $SbCl_5$	0 minutes	RO normal	YO Broken tone
4	0.5	0.80 $SbCl_5$	0 minutes	RO normal	O normal
5	0.5	1.00 $SbCl_5$	0 minutes	RO normal	O normal
6	0.5	1.50 $SbCl_5$	0 minutes	RO normal	O normal
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of concentration of Apocholic Acid

1	0.01	0.20 $SbCl_3$	3 minutes	Y Tint #1	Colorless
2	0.05	0.20 $SbCl_3$	3 minutes	Y Tint #1	Colorless
3	0.10	0.20 $SbCl_3$	3 minutes	OY normal	Colorless
4	0.30	0.20 $SbCl_3$	3 minutes	OY normal	Colorless

SERIES G-1 Continued

Effect of concentration of Apocholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
5	0.80	0.20 $SbCl_3$	3 minutes	OY normal *	Y Tint #2
6	1.00	0.20 $SbCl_3$	3 minutes	OY normal *	Y Tint #2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.01	0.05 $SbCl_5$	0 minutes	YO Tint #2	Colorless
2	0.05	0.05 $SbCl_5$	0 minutes	OY Shade 1	Colorless
3	0.10	0.05 $SbCl_5$	0 minutes	RO normal	Colorless
4	0.30	0.05 $SbCl_5$	0 minutes	RO normal	Colorless
5	0.80	0.05 $SbCl_5$	0 minutes	RO normal	Colorless
6	1.00	0.05 $SbCl_5$	0 minutes	RO normal	BG normal
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of time of heating

1	0.10	0.10 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.10	0.10 $SbCl_3$	3 minutes	Y normal	Colorless

* Red ppt. (OR Shade 2) formed on standing insoluble in $CHCl_3$.

SERIES G-1 (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	Color diluted
3	0.10	0.10 $SbCl_3$	6 minutes	OY Shade 1	Colorless
4	0.10	0.10 $SbCl_3$	10 minutes	OY Shade 1	Colorless*
5	0.10	0.10 $SbCl_3$	15 minutes	OY Shade 1	Colorless*
6	0.10	0.10 $SbCl_3$	20 minutes	YO Shade 1	Colorless*
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.10	0.05 $SbCl_5$	0 minutes	Colorless	Colorless
2	0.10	0.05 $SbCl_5$	3 minutes	YO Shade 2	Y Tint #2
3	0.10	0.05 $SbCl_5$	6 minutes	YO Broken tone	Y Tint #2
4	0.10	0.05 $SbCl_5$	10 minutes	OY Shade 2	Y Tint #2
5	0.10	0.05 $SbCl_5$	15 minutes	OY Shade 2 apprx.	OY Shade 2
6	0.10	0.05 $SbCl_5$	20 minutes	OY Shade 2 apprx.	OY Shade 2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

* These samples yielded an insoluble yellow oil.

SERIES G-2

A P O C H O L I C A C I D

Solution of Apocholic Acid in 95% alcohol. Each cc. contains 0.400 mgs. Alcoholic solutions of antimony reagents used in this run.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.01 $SbCl_3$	5 minutes	Y Shade 1	Colorless
2	0.1	0.05 $SbCl_3$	5 minutes	Y Shade 1	Colorless
3	0.1	0.10 $SbCl_3$	5 minutes	Y Sahde 1	Colorless
4	0.1	0.15 $SbCl_3$	5 minutes	Y normal	Colorless
5	0.1	0.20 $SbCl_3$	5 minutes	Y normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY			
1	0.1	0.01 $SbCl_5$	5 minutes	Y Shade 2	Colorless
2	0.1	0.05 $SbCl_5$	5 minutes	Y Shade 1	Colorless
3	0.1	0.10 $SbCl_5$	5 minutes	Y normal	Colorless
4	0.1	0.15 $SbCl_5$	5 minutes	Y normal	Colorless

SERIES G-2 (Continued)

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
5	0.10	0.20 $SbCl_5$	5 minutes	Y Normal	Colorless

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY

Effect of concentration of Apocholic Acid

1	0.01	0.20 $SbCl_3$	5 minutes	Colorless	Colorless
2	0.05	0.20 $SbCl_3$	5 minutes	Y Tint #1	Colorless
3	0.10	0.20 $SbCl_3$	5 minutes	OY normal	Colorless
4	0.20	0.20 $SbCl_3$	5 minutes	OY normal	Colorless
5	0.40	0.20 $SbCl_3$	5 minutes	OY normal	Colorless

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY

1	0.01	0.20 $SbCl_5$	5 minutes	Y Tint #2	Colorless
2	0.05	0.20 $SbCl_5$	5 minutes	Y Tint #2	Colorless
3	0.10	0.20 $SbCl_5$	5 minutes	Y Tint #1	Colorless

SERIES G-2 (continued)

Effect of concentration of Apocholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
4	0.20	0.20 $SbCl_5$	5 minutes	OY normal	Colorless
5	0.40	0.20 $SbCl_5$	5 minutes	OY normal	Y Tint #2

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY

Effect of time of heating

1	0.20	0.20 $SbCl_3$	1 minute	Colorless	Colorless
2	0.20	0.20 $SbCl_3$	5 minutes	RV normal*	RV normal*
3	0.20	0.20 $SbCl_3$	10 minutes	OY normal	Colorless
4	0.20	0.20 $SbCl_3$	15 minutes	OY normal	Colorless
5	0.20	0.20 $SbCl_3$	25 minutes	Y Shade 2	Colorless

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY

1	0.20	0.20 $SbCl_5$	1 minute	Colorless	Colorless

* This test was checked three times and in all cases produced a yellow color (Y normal) and is believed to be due to a trace of Ergosterol picked up while

SERIES G-2 (Continued)

Effect of time of heating

* (Continued) giving a student a sample of purified Ergosterol for a melting point standard.

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
2	0.20	0.20 $SbCl_5$	5 minutes	Y normal	Colorless
3	0.20	0.20 $SbCl_5$	10 minutes	OY normal	Colorless
4	0.20	0.20 $SbCl_5$	15 minutes	OY Shade 1 *	Turbid
5	0.20	0.20 $SbCl_5$	25 minutes	OY Shade 2 *	Turbid

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY

* The turbidity in this case was not removed by HCl.

SERIES H

C H O L I C A C I D

Solution of Cholic Acid in 95% alcohol. Each cc. contains 10.253 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.05	0.01 $SbCl_3$	1 minute	GY normal	Colorless
2	0.05	0.03 $SbCl_3$	1 minute	OY normal	Colorless *
3	0.05	0.05 $SbCl_3$	1 minute	Y normal	Colorless *
4	0.05	0.10 $SbCl_3$	1 minute	Y normal	Colorless *
5	0.05	0.20 $SbCl_3$	1 minute	Y normal	Colorless *
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.05	0.01 $SbCl_5$	1 minute	Colorless	Colorless
2	0.05	0.03 $SbCl_5$	1 minute	Black-brown	Black residue
3	0.05	0.05 $SbCl_5$	1 minute	Black-brown	YO Shade 1
4	0.05	0.10 $SbCl_5$	1 minute	Black-brown	YO Shade 1
5	0.05	0.20 $SbCl_5$	1 minute	Black-brown	YO normal

* Samples yielded insoluble red ppt. (R normal). On standing ppt. changed to GB.

SERIES H (Continued)

Effect of concentration of Cholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.20 $SbCl_3$	4 minutes	OY normal	GY Tint #2
2	0.05	0.20 $SbCl_3$	4 minutes	OY normal	Colorless
3	0.10	0.20 $SbCl_3$	4 minutes	OY normal	Y normal[1]
4	0.15	0.20 $SbCl_3$	4 minutes	OY normal	Y normal [2]
5	0.20	0.20 $SbCl_3$	4 minutes	OY normal	Y normal [3]
6	0.21	0.20 $SbCl_3$	3 1/2 minutes	YO normal	GY normal [4]
7	0.25	0.20 $SbCl_3$	3 1/2 minutes	YO normal	GY normal
8	0.30	0.20 $SbCl_3$	3 1/2 minutes	YO normal	GY normal
9	0.35	0.20 $SbCl_3$	3 1/2 minutes	YO normal	GY normal
10	0.40	0.20 $SbCl_3$	3 1/2 minutes	YO normal	GY normal

1. On standing for 15 minutes RV ppt. forms which if dissolved in alcohol and concentrated HCl yields deep blue fading to GY Tint #1.
2. On adding HCl and alcohol color becomes G broken tone.
3. On standing for 15 minutes color becomes YG Shade 1.
4. Samples 6-10 inclusive yield insoluble red ppt. RO normal if HCl and alcohol added top layer becomes GB Tint #1, bottom layer G Tint #1. If ppt. stands over-night color becomes blue which dissolves in alcoholic HCl to give G broken.

SERIES H (Continued)

Effect of concentration of Cholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.20 $SbCl_5$	0 minutes	OR normal	OY Tint #1
2	0.05	0.20 $SbCl_5$	0 minutes	OR normal	YO broken tone
3	0.10	0.20 $SbCl_5$	0 minutes	OR normal	YO broken tone
4	0.15	0.20 $SbCl_5$	0 minutes	RO Shade 1	OY Shade 2
5	0.20	0.20 $SbCl_5$	0 minutes	RO Shade 1	OY Shade 2

On standing over-night all samples yielded a blue residue which dissolved in alcoholic HCl to give G broken tone.

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Effect of time of heating

1	0.10	0.20 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.10	0.20 $SbCl_3$	3 minutes	YO normal	Colorless
3	0.10	0.20 $SbCl_3$	6 minutes	YO normal	Colorless
4	0.10	0.20 $SbCl_3$	9 minutes	YO normal	Colorless
5	0.10	0.20 $SbCl_3$	12 minutes	YO normal	Colorless

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES H (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.10	0.20 $SbCl_5$	0 minutes	OR normal	GY Shade 2
2	0.10	0.20 $SbCl_5$	3 minutes	O Shade 2	YO normal
3	0.10	0.20 $SbCl_5$	6 minutes	O Shade 2	YO normal
4	0.10	0.20 $SbCl_5$	9 minutes	O Shade 2	YO normal
5	0.10	0.20 $SbCl_5$	12 minutes	O Shade 2	YO normal

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

SERIES J

G L Y C O C H O L I C A C I D

Solution of Glycocholic Acid in 95% alcohol. Each cc. contains 0.596 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.10	0.05 $SbCl_3$	6 minutes	Y normal	Colorless
2	0.10	0.10 $SbCl_3$	6 minutes	OY Shade 1	Colorless
3	0.10	0.20 $SbCl_3$	6 minutes	OY Shade 1	Colorless
4	0.10	0.40 $SbCl_3$	6 minutes	Y Tint #2	Colorless
5	0.10	0.60 $SbCl_3$	6 minutes	Y Tint #2	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH HAD BEEN ADDED 0.1 CC. HCl (conc.) TO REMOVE TURBIDITY			
1	0.10	0.05 $SbCl_5$	6 minutes	OY Shade 2	Colorless
2	0.10	0.10 $SbCl_5$	6 minutes	OY Shade 1	Colorless
3	0.10	0.20 $SbCl_5$	6 minutes	OY normal	Colorless
4	0.10	0.40 $SbCl_5$	6 minutes	Y Tint #2	Colorless
5	0.10	0.60 $SbCl_5$	6 minutes	GY Tint #2	Colorless

SERIES J (Continued)

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH
HAD BEEN ADDED 0.1 CC. HCl (conc.)
TO REMOVE TURBIDITY

ALCOHOLIC SOLUTIONS OF ANTIMONY REAGENTS USED IN THIS SERIES.

Effect of concentration of Glycocholic Acid

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.20 $SbCl_3$	3 minutes	Colorless	Colorless
2	0.05	0.20 $SbCl_3$	3 minutes	Y Tint #2	Colorless
3	0.10	0.20 $SbCl_3$	3 minutes	Y Tint #2	Colorless
4	0.20	0.20 $SbCl_3$	3 minutes	Y Tint #1	Colorless
5	0.40	0.20 $SbCl_3$	3 minutes	Y Tint #1	Colorless

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH
HAD BEEN ADDED 0.1 CC. HCl (conc.)
TO REMOVE TURBIDITY

1	0.01	0.20 $SbCl_5$	3 minutes	Y Tint #2	Colorless
2	0.05	0.20 $SbCl_5$	3 minutes	Y Tint #2	Colorless
3	0.10	0.20 $SbCl_5$	3 minutes	Y Tint #1	Colorless
4	0.20	0.20 $SbCl_5$	3 minutes	OY normal	Colorless
5	0.40	0.20 $SbCl_5$	3 minutes	OY normal	Colorless

SERIES J (continued)

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH
HAD BEEN ADDED 0.1 CC. HCl (conc.)
TO REMOVE TURBIDITY

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.20	0.20 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.20	0.20 $SbCl_3$	6 minutes	OY normal	Colorless
3	0.20	0.20 $SbCl_3$	10 minutes	OY normal	Colorless
4	0.20	0.20 $SbCl_3$	15 minutes	OY Shade 1	Colorless
5	0.20	0.20 $SbCl_3$	25 minutes	OY Shade 1	Colorless

ALL SAMPLES DILUTED WITH 2 CC. C_2H_5OH TO WHICH
HAD BEEN ADDED 0.1 CC. HCl (conc.)
TO REMOVE TURBIDITY

1	0.20	0.20 $SbCl_5$	0 minutes	Colorless	Colorless
2	0.20	0.20 $SbCl_5$	6 minutes	OY normal	GY Tint #2
3	0.20	0.20 $SbCl_5$	10 minutes	OY Shade 1	Y Tint #2
4	0.20	0.20 $SbCl_5$	15 minutes	Y Shade 1	Y Tint #2
5	0.20	0.20 $SbCl_5$	25 minutes	Y Shade 2	Y Tint #2

ALL SAMPLES DILUTED AS ABOVE

SERIES K

TESTOSTERONE ACETATE

Solution of Testosterone Acetate in $CHCl_3$. Each cc. contains 0.758 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.10	0.01 $SbCl_5$	5 minutes	Colorless	Colorless
2	0.10	0.08 $SbCl_5$	5 minutes	OY Tint #2	Colorless
3	0.10	0.12 $SbCl_5$	5 minutes	OY Tint #2	Colorless
4	0.10	0.20 $SbCl_5$	5 minutes	OY Tint #2	Colorless
5	0.10	0.30 $SbCl_5$	5 minutes	OY Tint #2	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.10	0.01 $SbCl_5$	5 minutes	YO Shade 1	Y Tint #1
2	0.10	0.08 $SbCl_5$	5 minutes	YO Shade 1	Turbid
3	0.10	0.10 $SbCl_5$	5 minutes	YO Shade 2	Turbid
4	0.10	0.15 $SbCl_5$	5 minutes	YO Shade 2	Y Tint #1
5	0.10	0.25 $SbCl_5$	5 minutes	YO Shade 2	Y Tint #1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

SERIES K (Continued)

Effect of concentration of Testosterone Acetate

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.10 $SbCl_3$	5 minutes	OY Tint #1	Colorless
2	0.05	0.10 $SbCl_3$	5 minutes	OY Tint #1	Colorless
3	0.10	0.10 $SbCl_3$	5 minutes	OY Tint #1	Colorless
4	0.15	0.10 $SbCl_3$	5 minutes	OY Tint #1	Colorless
5	0.20	0.10 $SbCl_3$	5 minutes	OY Tint #1	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.01	0.10 $SbCl_5$	5 minutes	YO Shade 1	Colorless
2	0.05	0.10 $SbCl_5$	5 minutes	YO Shade 1	Y Tint #2
3	0.10	0.10 $SbCl_5$	5 minutes	YO Shade 2	Y Tint #2
4	0.15	0.10 $SbCl_5$	5 minutes	YO Shade 1	Y Tint #1
5	0.20	0.10 $SbCl_5$	5 minutes	YO Shade 1	Y Tint #1
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of time of heating

1	0.10	0.20 $SbCl_3$	3 minutes	OY Tint #2	Colorless

SERIES K (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
2	0.10	0.20 $SbCl_3$	5 minutes	OY Tint #1	Y Tint #2 Pale
3	0.10	0.20 $SbCl_3$	10 minutes	YO Tint #2	OY Tint #2*
4	0.10	0.20 $SbCl_3$	15 minutes	O Tint #2	Y Tint #2*
5	0.10	0.20 $SbCl_3$	20 minutes	O Tint #2	YO Tint #2*

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

Tubes 3,4 and 5, on standing, for about 1 hour yield a blue liquid GB Tint #2 and a strong red fluorescence.

1	0.10	0.20 $SbCl_5$	1 minute	YO Shade 1	OY Tint #1
2	0.10	0.20 $SbCl_5$	3 minutes	YO Shade 2	Y Tint #1
3	0.10	0.20 $SbCl_5$	5 minutes	YO Shade 2	Y Tint #1
4	0.10	0.20 $SbCl_5$	10 minutes	YO Shade 2	Turbid
5	0.10	0.20 $SbCl_5$	20 minutes	YO Shade 1	Turbid

ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$

* Green fluorescence on first dilution.

SERIES K (Continued)

Special tests using alcoholic antimony reagents

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.10	0.40 $SbCl_3$	29 minutes	GY broken tone	Blue*
2	0.10	0.32 $SbCl_5$	20 minutes	YO Shade 2	Turbid
Special tests heating to 150°C					
1	0.20	0.20 $SbCl_3$	5 minutes	Blue ppt.	Blue*

Blue residue partially soluble in $CHCl_3$ giving a pale blue liquid with an intense red fluorescence. On adding alcohol cautiously solution becomes clear blue though still fluorescent. If more alcohol is added color finally fades to yellow (GY normal).

* Intense red fluorescence.

SERIES L

SPECIAL RUN AT 150°C

cc. soln.	cc. reagent	color undiluted	color diluted	color with C_2H_5OH	color with HCl
0.1 testerone acetate	0.2 $SbCl_3$	RV Shade 2	BV Tint #1	GB normal	B Shade 1
0.1 oestrone	0.2 $SbCl_3$	VR normal	VR Tint #2	OR Tint #2	VR Tint #2
0.1 cholic acid	0.2 $SbCl_3$	YO normal	YO Shade 1	YO Shade 1	O Shade 1
0.1 cholesterol	0.2 $SbCl_3$	O Shade 2	Turbid	Turbid	Colorless
0.1 ergosterol	0.2 $SbCl_3$	YO Shade 2	Turbid	Turbid	Colorless
0.1 phenanthrene	0.2 $SbCl_3$	OY Shade 2	Turbid	Turbid	Colorless
0.1 dehydrocholic acid	0.2 $SbCl_3$	OY Shade 2	Turbid	Turbid	Colorless
0.1 desoxycholic acid	0.2 $SbCl_3$	YO Shade 2	Turbid	Turbid	Colorless
0.1 apocholic acid	0.2 $SbCl_3$	YO Shade 2	Turbid	Turbid	Colorless
0.1 glycocholic acid	0.2 $SbCl_3$	YO Broken tone	Turbid	Turbid	Colorless

In carrying out these tests the following procedure was used:

(a) Samples were first diluted with 1 cc. chloroform. (color diluted)
(b) 0.5 cc. of 95% alcohol was then added. (color with C_2H_5OH)
(c) 0.5 cc. of conc. HCl finally added. (color with HCl)

SERIES M-1

SPECIAL TESTS ON ERGOSTEROL AND CHOLESTEROL USING THE ANTIMONY REAGENTS AND THE ROSENHEIM REAGENT

In all tests 0.1 cc. of cholesterol solution was used equivalent to 0.1308 mgs. and 0.43 cc. of ergosterol solution was used equivalent to 0.1298 mgs.

In these tests the alcohol was first evaporated by heating just to dryness on a water bath.

Cholesterol Tests

Tube	cc. soln.	cc. reagent	color (undiluted)	color diluted	heating mins.
1	0.1	0.5 $SbCl_3$	Colorless	Colorless	0 minutes
2	0.1	0.22 $SbCl_5$	Sherry wine shade	YO Broken tone	0 minutes
3	0.1	50 drops Rosenheim Reagent	Colorless	Colorless	0 minutes

On standing overnight cholesterol and $SbCl_3$ yeilds a purple color (VR Tint 2)

Ergosterol Tests

1	0.43	0.5 $SbCl_3$	R Tint #2	B Tint #2	0 minutes
2	0.43	0.22 $SbCl_5$	RO Shade 1	VR Shade 1	0 minutes
3	0.43	50 drops Rosenheim Reagent	Colorless	Colorless	0 minutes

SERIES M-2

SPECIAL TESTS ON ERGOSTEROL IN CHLOROFORM SOLUTION USING THE ANTIMONY REAGENTS AND THE ROSENHEIM REAGENT

In these tests 0.5 cc. of a solution of ergosterol in $CHCl_3$ was used. Each cc. contains 0.228 mgs. of ergosterol.

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted	color after one hour
1	0.5	15 drops Rosenheim Reagent*	0 minutes	Colorless	Colorless	GY Tint #2
2	0.5	0.22 $SbCl_5$	0 minutes	0 normal	R Tint #1	VR Shade 1
3	0.5	0.50 $SbCl_5$	0 minutes	R Tint #2	RV Tint #2	Colorless

* No color developed with the Rosenheim test on standing for 15 minutes. At that time 15 drops more of the reagent was added yeilding the color after one hour.

SERIES N

COMPARATIVE TESTS ON APOCHOLIC AND CHOLIC ACIDS

Tube	cc. soln.	cc. reagent	heating mins.	color on adding HCl	color after one hour	color overnight
1	7.24 cc. apocholic acid	14.2 cc. conc. HCl	0 minutes	colorless	Y Tint #2	Y Tint #1
2	0.29 cc. cholic acid	0.56 cc. conc. HCl	0 minutes	colorless	colorless	Y Tint #1

The above concentrations were used to yeild a final HCl concentration of 25% as called for in the Hammarsten reaction.

SERIES O

EFFECT OF SOLVENT ON COLOR PRODUCED WITH ANTIMONY REAGENTS

In this series an equal number of mols of test substance was treated in all cases with the same amount of antimony chloride irregardless of the solvent used.

Substance tested	cc. soln. containing equal number of mols.
apocholic acid	0.724 cc.
cholesterol	0.218 cc.
dehydrocholic acid	1.06 cc.
desoxycholic acid	1.17 cc.
ergosterol	1.00 cc.
glycocholic acid	0.56 cc.
oestrone	2.00 cc.
phenanthrene	0.532 cc.
testosterone acetate	0.322 cc.
cholic acid	0.029 cc.

In all cases the solvent was removed by evaporating on a water bath just to dryness.

SERIES O (Continued)

TESTS USING ANTIMONY TRICHLORIDE IN CHLOROFORM

In this and all subsequent tests in this series the number of cc. of test substance used is that indicated on the previous page.

Substance tested	cc. reagent	color on adding	color overnight
Apocholic acid	0.8 cc. $SbCl_3$	Colorless	GY normal*
Cholesterol	0.8 cc. $SbCl_3$	Y normal	O normal
Dehydrocholic Acid	0.8 cc. $SbCl_3$	Colorless	Colorless
Desoxycholic Acid	0.8 cc. $SbCl_3$	Colorless	GY normal*
Ergosterol	0.8 cc. $SbCl_3$	O Shade 1	R Tint #1*
Glycocholic Acid	0.8 cc. $SbCl_3$	Y normal*	GY normal*
Oestrone	0.8 cc. $SbCl_3$	Y Tint #2	R Tint #1*
Phenanthrene	0.8 cc. $SbCl_3$	Colorless	Colorless
Testosterone Acetate	0.8 cc. $SbCl_3$	Colorless	GY Tint #2
Cholic Acid	0.8 cc. $SbCl_3$	Y normal*	GY normal*

TESTS USING ANTIMONY PENTACHLORIDE IN CHLOROFORM

Apocholic Acid	0.5 $SbCl_5$	Sherry wine shade	YO Shade 1

* These samples yeilded a colored oil and a clear supernatent liquor.

SERIES O (Continued)

Substance tested	cc. reagent	color on adding	color overnight
Phenanthrene	2.47 cc. $SbCl_3$	Colorless	Colorless
Testosterone Acetate	2.47 cc. $SbCl_3$	Colorless	Colorless
Oestrone	2.47 cc. $SbCl_3$	Colorless	Colorless
Cholesterol	2.47 cc. $SbCl_3$	Colorless	Colorless
Ergosterol	2.47 cc. $SbCl_3$	Colorless	GY Tint #2
	TESTS USING ANTIMONY PENTACHLORIDE IN ALCOHOL		
Apocholic Acid	0.81 cc. $SbCl_5$	Colorless	Colorless
Cholic Acid	0.81 cc. $SbCl_5$	Colorless	Colorless
Dehydrocholic Acid	0.81 cc. $SbCl_5$	Colorless	Colorless
Desoxycholic Acid	0.81 cc. $SbCl_5$	Colorless	Colorless
Glycocholic Acid	0.81 cc. $SbCl_5$	Colorless	Colorless
Phenanthrene	0.81 cc. $SbCl_5$	Colorless	Colorless
Testosterone Acetate	0.81 cc. $SbCl_5$	Colorless	Colorless
Oestrone	0.81 cc. $SbCl_5$	Colorless	Y Tint #2
Cholesterol	0.81 cc. $SbCl_5$	Colorless	Colorless
Ergosterol	0.81 cc. $SbCl_5$	GY Tint #1	OY Shade 1

SERIES O (Continued)

Substance tested	cc. reagent	color on adding	color overnight
Cholic Acid	0.5 cc. $SbCl_5$	Sherry wine shade	YO Shade 1
Dehydrocholic Acid	0.5 cc. $SbCl_5$	GY Tint #1	GY Tint #2
Desoxycholic Acid	0.5 cc. $SbCl_5$	YO Shade 1	YO Shade 2
Glycocholic Acid	0.5 cc. $SbCl_5$	OY Shade 2	YO Shade 1
Phenanthrene	0.5 cc. $SbCl_5$	Black-brown	OY Shade 2
Testosterone Acetate	0.5 cc. $SbCl_5$	Y Tint #1	OY Shade 1
Oestrone	0.5 cc. $SbCl_5$	O Shade 2	YO Shade 2
Cholesterol	0.5 cc. $SbCl_5$	OR Shade 2	OY Shade 2
Ergosterol	0.5 cc. $SbCl_5$	R Shade 2	R normal
	TESTS USING ANTIMONY TRICHLORIDE IN ALCOHOL		
Apocholic Acid	2.47 cc. $SbCl_3$	Colorless	Colorless
Cholic Acid	2.47 cc. $SbCl_3$	Colorless	Colorless
Dehydrocholic Acid	2.47 cc. $SbCl_3$	Colorless	Colorless
Desoxycholic Acid	2.47 cc. $SbCl_3$	Colorless	Colorless
Glycocholic Acid	2.47 cc. $SbCl_3$	Colorless	Colorless

SERIES O (Continued)

TESTS USING ANTIMONY TRICHLORIDE IN GLACIAL ACETIC ACID

Substance Tested	cc. reagent	color on adding	color overnight
Apocholic Acid	2.33 cc. $SbCl_3$	Colorless	Colorless
Cholic Acid	2.33 cc. $SbCl_3$	"	"
Dehydrocholic Acid	2.33 cc. $SbCl_3$	"	"
Desoxycholic Acid	2.33 cc. $SbCl_3$	"	"
Glycocholic Acid	2.33 cc. $SbCl_3$	"	"
Phenanthrene	2.33 cc. $SbCl_3$	"	"
Testosterone Acetate	2.33 cc. $SbCl_3$	"	"
Oestrone	2.33 cc. $SbCl_3$	"	"
Cholesterol	2.33 cc. $SbCl_3$	"	"
Ergosterol	2.33 cc. $SbCl_3$	"	"

TESTS USING ANTIMONY PENTACHLORIDE IN GLACIAL ACETIC ACID

Apocholic Acid	0.30 cc. $SbCl_5$	YO Tint #1	Y Shade 1
Cholic Acid	0.30 cc. $SbCl_5$	YO Tint #2	Y Shade 1
Dehydrocholic Acid	0.30 cc. $SbCl_5$	Colorless	Y Tint #2

SERIES O (Continued)

Substance Tested	cc. reagent	color on adding	color overnight
Desoxycholic Acid	0.30 cc. $SbCl_5$	Colorless	GY Tint #1
Glycocholic Acid	0.30 cc. $SbCl_5$	Colorless	OY Shade 1
Phenanthrene	0.30 cc. $SbCl_5$	Colorless	Colorless
Testosterone Acetate	0.30 cc. $SbCl_5$	Colorless	Colorless
Oestrone	0.30 cc. $SbCl_5$	OY normal	Y Shade 2
Cholesterol	0.30 cc. $SbCl_5$	GB Tint #2	BG Shade 2
Ergosterol	0.30 cc. $SbCl_5$	B Shade 1	VB Shade 2

SERIES P

VITAMIN D_2

Solution of Vitamin D_2 in $CHCl_3$. Each cc. contains 0.239 mgs.

Effect of concentration of antimony reagent

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.01 $SbCl_3$	0 minutes	Colorless	Colorless
2	0.1	0.03 $SbCl_3$	0 minutes	OY Tint #2	Colorless
3	0.1	0.05 $SbCl_3$	0 minutes	YO Tint #2	Colorless
4	0.1	0.10 $SbCl_3$	0 minutes	OY normal	Colorless
5	0.1	0.20 $SbCl_3$	0 minutes	OY normal	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.01 $SbCl_5$	0 minutes	OY normal	Y Tint #2
2	0.1	0.03 $SbCl_5$	0 minutes	OY normal	Y Tint #2
3	0.1	0.05 $SbCl_5$	0 minutes	OY normal	Y Tint #2
4	0.1	0.10 $SbCl_5$	0 minutes	OY normal	YO Tint #2
5	0.1	0.20 $SbCl_5$	0 minutes	OY normal	O Tint #1
6	0.1	0.50 $SbCl_5$	0 minutes	OY normal	YO Tint #1

SERIES P (Continued)

Effect of concentration of Vitamin D_2

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.01	0.20 $SbCl_3$	0 minutes	YO Tint #2	Colorless
2	0.03	0.20 $SbCl_3$	0 minutes	YO Tint #1*	Colorless
3	0.05	0.20 $SbCl_3$	0 minutes	OY normal*	Colorless
4	0.10	0.20 $SbCl_3$	0 minutes	OY normal*	Colorless
5	0.20	0.20 $SbCl_3$	0 minutes	OY normal*	Colorless
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.01	0.50 $SbCl_5$	0 minutes	Y Tint #1*	Colorless
2	0.03	0.50 $SbCl_5$	0 minutes	OY normal*	YO Tint #2*
3	0.05	0.50 $SbCl_5$	0 minutes	YO normal*	YO Tint #2*
4	0.10	0.50 $SbCl_5$	0 minutes	YO normal*	O Tint #1*
5	0.20	0.50 $SbCl_5$	0 minutes	YO normal*	YO normal*
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

* Color indicated is closest match. All samples, however, show a slightly pinkish tone.

SERIES P (Continued)

Effect of time of heating

Tube	cc. soln.	cc. reagent	heating mins.	color (undiluted)	color diluted
1	0.1	0.20 $SbCl_3$	1 minute	YO Shade 1	Colorless
2	0.1	0.20 $SbCl_3$	3 minutes	OY Shade 1	YO Tint #2
3	0.1	0.20 $SbCl_3$	5 minutes	YO Shade 1	YO Tint #2
4	0.1	0.20 $SbCl_3$	10 minutes	YO Shade 1	YO Tint #2
5	0.1	0.20 $SbCl_3$	15 minutes	YO Shade 1	YO Tint #2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			
1	0.1	0.20 $SbCl_5$	1 minute	YO Broken tone	Y Tint #2
2	0.1	0.20 $SbCl_5$	3 minutes	YO Broken tone	Y Tint #2
3	0.1	0.20 $SbCl_5$	5 minutes	YO Broken tone	Y Tint #2
4	0.1	0.20 $SbCl_5$	10 minutes	YO Broken tone	Y Tint #2
5	0.1	0.20 $SbCl_5$	15 minutes	YO Broken tone	Y Tint #2
		ALL SAMPLES DILUTED WITH 2 CC. $CHCl_3$			

Effect of solvent on color produced with antimony reagents

					Color after 1 hour
1	0.1	0.8 $SbCl_3$ (in $CHCl_3$)	0 minutes	OY normal	YO Tint #1

SERIES P (Continued)

Effect of solvent on color produced with antimony reagents

Tube	cc. soln.	cc. reagent	heating mins.	color on adding	color after 1 hour
2	0.1	0.5 $SbCl_5$ (in $CHCl_3$)	0 minutes	YO normal	YO Shade 1
3	0.1	2.47 $SbCl_3$ (in C_2H_5OH)	0 minutes	Colorless	Colorless
4	0.1	0.81 $SbCl_5$ (in C_2H_5OH)	0 minutes	Colorless	GY Tint #2
5	0.1	2.33 $SbCl_3$ (in Glacial Acetic Acid)	0 minutes	Colorless	Colorless
6	0.1	0.3 $SbCl_5$ (in Glacial Acetic Acid)	0 minutes	Y Shade 1	GY Broken Tone

In these tests the chloroform was first removed by evaporating just to dryness on a water bath.

SERIES Q

SPECIAL TESTS ON HORMONES

Solution of Oestrone in 95% alcohol. Each cc. contains 0.1 mgs.

Solution of Oestradiol in 95% alcohol. Each cc. contains 0.042 mgs.

Solution of Oestriol in 95% alcohol. Each cc. contains 0.022 mgs.

Solution of Testosterone Acetate in $CHCl_3$. Each cc. contains 0.1516 mgs. (Ten cc. of original stock solution made up to 50 cc. in $CHCl_3$)

Preliminary Tests Using H_2SO_4 and Hormone Reagent

Treat with 0.1 cc. of concentrated H_2SO_4 on the water bath for two minutes. Cool, add 0.8 cc. H_2O and then five drops of the hormone reagent. Reheat on water bath for two minutes.

OESTRIOL	OESTRADIOL	OESTRONE
1.36 cc.	0.72 cc.	0.30 cc.
BG Tint #1	O Tint #2--	O Tint #2

Preliminary Tests Using Hormone Reagent Alone

Treat with 0.1 cc. of hormone reagent for four minutes. Cool, dilute with one cc. concentrated HCl, and add two cc. of H_2O.

1.36 cc.	0.72 cc.	0.30 cc.
Colorless	R Tint #2	R Tint #2

Effect of heating on oestrone test (This test uses the hormone reagent alone)

Tube	cc. of oestrone	cc. reagent	time of heating	color
1	0.1 cc.	0.05 cc.	10 minutes	OR Tint #2 (pale)
2	0.1 cc.	0.05 cc.	20 minutes	OR Tint #2
3	0.1 cc.	0.05 cc.	30 minutes	OR Tint #2 (strong)
Effect of concentration of reagent in oestrone test				
1.	0.1 cc.	0.07 cc.	30 minutes	OR Tint #2
2	0.1 cc.	0.10 cc.	30 minutes	OR Tint #2 (pale)
3	0.1 cc.	0.15 cc.	30 minutes	O Tint #2
Effect of concentration of oestrone				
1	0.01 cc.	0.05 cc.	30 minutes	Colorless
2	0.02 cc.	0.05 cc.	30 minutes	Colorless
3	0.03 cc.	0.05 cc.	30 minutes	Colorless
4	0.04 cc.	0.05 cc.	30 minutes	OR Tint #2
5	0.07 cc.	0.05 cc.	30 minutes	OR Tint #2
6	0.08 cc.	0.05 cc.	30 minutes	OR Tint #2 (strong)

SERIES Q (Continued)

Effect of concentration of oestriol (This test uses H_2SO_4 plus the hormone reagent.)

Tube	cc. of oestriol	cc. of reagent	time of heating	color
1	0.08 cc.	3 drops	See footnote	Colorless
2	0.20 cc.	3 drops	See footnote	Colorless
3	0.36 cc.	3 drops	See footnote	Colorless
4	0.42 cc.	3 drops	See footnote	Just visible
5	0.46 cc.	3 drops	See footnote	BG Tint #2
6	0.92 cc.	3 drops	See footnote	BG Tint #1
7	1.36 cc.	3 drops	See footnote	BG Tint #1

NOTE: In this test the hormones are first heated with 0.1 cc. of H_2SO_4 (conc.) for three minutes on a water bath. On cooling 0.5 cc. H_2O is added and 3 drops of the reagent introduced. The tube is then reheated for seven minutes.

SERIES Q (Continued)

Effect of concentration of testosterone acetate. (This test uses the hormone reagent alone).

Tube	cc. of testosterone acetate	cc. reagent	time of heating	color
1	0.02 cc.	0.05 cc.	30 minutes	Colorless
2	0.04 cc.	0.05 cc.	30 minutes	Colorless
3	0.06 cc.	0.05 cc.	30 minutes	Colorless
4	0.08 cc.	0.05 cc.	30 minutes	BG Tint #2
5	0.10 cc.	0.05 cc.	30 minutes	BG Tint #2
6	0.14 cc.	0.05 cc.	30 minutes	BG Tint #1
7	0.18 cc.	0.05 cc.	30 minutes	BG Tint #1

NOTE: In this test the sample is heated for thirty minutes with 0.05 cc. of the hormone reagent on the water bath. When cool 0.5 cc. of water and seven drops of concentrated HCl are added.

SERIES Q (Continued)

Effect of concentration of testosterone acetate (This test uses H_2SO_4 plus the hormone reagant)

Tube	cc. of testosterone acetate	cc. reagent	time of heating	color
1	0.02 cc.	3 drops	See footnote	Colorless
2	0.04 cc.	3 drops	See footnote	Colorless
3	0.06 cc.	3 drops	See footnote	Colorless
4	0.08 cc.	3 drops	See footnote	G Tint#2
5	0.10 cc.	3 drops	See footnote	G Tint#2
6	0.14 cc.	3 drops	See footnote	G Tint#1
7	0.20 cc.	3 drops	See footnote	G Tint#1

NOTE: In this test the hormones are first heated with 0.1 cc. of H_2SO_4 (conc.) for three minutes on a water bath. On cooling 0.5 cc. H_2O is added and 3 drops of the reagent introduced. The tube is then reheated for seven minutes.

GENERAL AND

THEORETICAL

Before considering the reactions of the antimony reagents with the substances tested it may advisable to consider, at the start, something of the nature of the antimony chlorides themselves as an aid in interpreting some of the observed facts. Both of the chlorides are extremely hygroscopic. This was found to be a very annoying factor in attempting to determine the melting point of the compound and is possibly the reason for the various melting points recorded in the literature. The solid trichloride if exposed to the air picks up more than its own weight of water in about 70 days. (Cf. Mellor, page 472). The trichloride is readily hydrolyzed by water yielding a turbid, acid, liquor formerly called "Liquor stibii muriatici".

$$SbCl_3 + H_2O = SbOCl + 2\ HCl$$

The turbidity readily disappears with the addition of HCl in accordance with the Law of Mass Action. H. von Wartenberg used this reaction to demonstrate this law. (Cf. Mellor, page 472.)

The liquid pentachloride fumes vigorously when exposed to the air, undergoing hydrolysis and depositing a white oxychloride. With HCl the pentachloride easily forms an addition compound which is stable in aqueous solution.

$$H^+Cl^- + SbCl_5 = H^+ + SbCl_6^-$$

This latter reaction has been substantiated by the fact that on adding silver nitrate there is no immediate precipitation of silver chloride. (Cf. Mellor, page 490.)

Of particular significance in regard to the pentachloride is its ability to function as a catalyst in organic chlorination. Thus Zetter[53] prepared the yellow tetrachlorphenanthrene by the action of the pentachloride on dry phenanthrene at room temperature. This fact must be borne in mind when considering the action of the pentachloride reagent. It will be noted that in all instances when the substance tested was heated with the pentachloride a yellow color resulted, even with those substances, e.g. ergosterol, that produced a blue or red color in the cold. The use of the pentachloride reagent is thus limited by its ability to form a stable, distinctive colored, soluble, complex in the cold. Such a conclusion is entirely in accordance with the present accepted mechanism of chlorination with metallic chlorides. Thus, Groggins[13] in his discussion of catalytic halogenation points out that in the chlorination of acetylene, using $SbCl_5$, an equimolecular compound of the acetylene and $SbCl_5$ is first formed which then breaks down into $SbCl_5$ and tetrachlorethane. The extra chlorine required is produced by the dissociation of an excess pentachloride. (Cf. Groggins, page 170).

$$C_2H_2 + SbCl_5 = C_2H_2.SbCl_5$$
$$2\ SbCl_5 = 2\ SbCl_3 + 2\ Cl_2$$
$$2\ Cl_2 + C_2H_2.SbCl_5 = C_2H_2Cl_4 + SbCl_5$$

A further point of consideration is that below 100°C $SbCl_5$ is without effect on the saturated hydrocarbons, since no chlorination takes place by substitution. (Cf. Groggins, page 170).

Consequently the saturated bile acids should produce no color. However, cholic acid readily undergoes dehydrogenation in the presence of the HCl always occurring in the antimony reagents and hence has an unsaturated linkage available for the formation of a colored complex.

It is evident that in order to obtain a test the compound must possess an unsaturated linkage either actual or potential, e.g. cholic acid.

Schoernheimer[38] has shown that the sterol color reactions are only applicable to the unsaturated sterols.

It will be noted that the heating limitation in regard to the pentachloride does not apply to the trichloride which in certain tests, e.g. sex hormones, requires fairly strong heating. In the latter case another property is possibly producing the effect, namely the dehydrating action of the fused trichloride. However, for the sake of completeness and because of the possibility that the combined effect of chlorinating and dehydrating might yield a distinctive color test samples were heated with

the pentachloride reagent as well as with the trichloride.

As has been shown above on heating the $SbCl_5$ dissociates into $SbCl_3$ and Cl_2 so that it can exert both a chlorinating and dehydrating action.

ERGOSTEROL & CHOLESTEROL

The following are the most important sterol color reactions. It will be noted that in all cases a strong dehydrating agent is required.

Lieberman-Burchard[20]

A solution of the sterol in chloroform is treated with concentrated H_2SO_4 and acetic anhydride.

Salkowski[36]

A solution of the sterol in chloroform is treated with concentrated H_2SO_4.

Lifschutz [21]

The sterol in glacial acetic acid is heated with perbenzoic acid and sulphuric acid is added.

Tschugajeff[42]

The sterol in glacial acetic acid is heated with zinc chloride and acetyl chloride.

Whitby[45]

The sterol in chloroform, or glacial acetic acid, is treated with concentrated H_2SO_4 containing CH_2O.

Rosenheim[32]

The sterol in chloroform is treated with a concentrated aqueous solution of CCl_3COOH.

Carr & Price[6]

The sterol in chloroform is treated with

a saturated solution of $SbCl_3$ in chloroform.

Rosenheim & Drummond[33]

The sterol in chloroform is treated with a solution of $AsCl_3$ in chloroform.

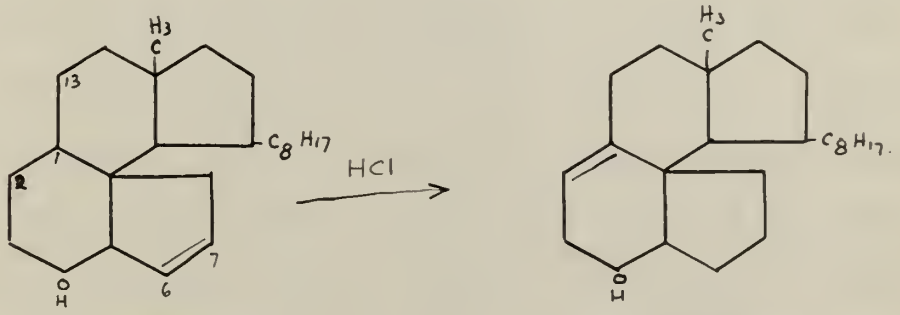
Steinle & Kahlenberg[40]

The sterol in chloroform is treated with a solution of $SbCl_5$ in $CHCl_3$.

With regard to the reactions of the antimony reagents and the sterols the most significant findings were the ability to differentiate ergosterol and cholesterol. Using the trichloride in a chloroform solution it was found that on treating ergosterol a red color (R Tint #2) developed at once, while with practically the same weight of cholesterol (the samples tested containing 0.1308 mgs. of cholesterol and 0.1298 mgs. of ergosterol) no color was obtained. Furthermore it was possible, though not so readily, to distinguish between these sterols by means of the pentachloride reagent. The cholesterol and the pentachloride yielded a yellow color (YO broken tone) while the ergosterol and the pentachloride yielded a very deep violet (VR shade 1) which gradually changed after about thirty minutes to a bluer violet (V broken tone). (See Series M-1).

The ability to distinguish between these sterols

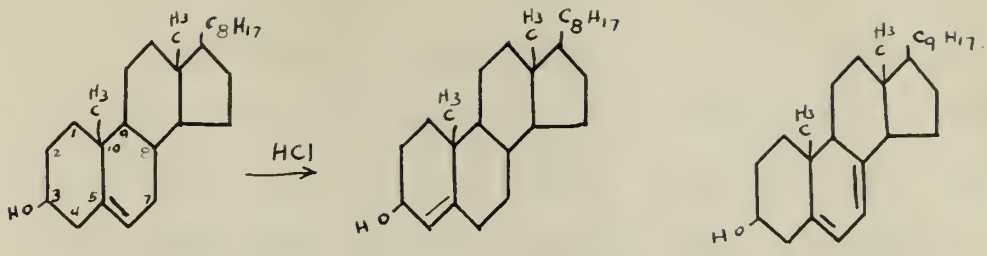
is generally attributed to the Rosenheim reaction.[32] In complete accord with the findings of Rosenheim it was noted that on heating, or on standing for about 14 hours, the cholesterol likewise gave the color reaction with the trichloride. This was accounted for by Rosenheim by the isomeric shift in the double bond that occurs when cholesterol is treated with HCl, forming allo-cholesterol. This shift had been demonstrated by Windaus[48] in 1927.



cholesterol allo-cholesterol

It will be noted that at the time that Rosenheim published his findings, 1929, the old formula for the sterols was in vogue and he accounted for the color as being characteristic for a double bond at $C_{1,2}$, or $C_{1,13}$. (the exact location of this bond was still undetermined but known to be at either of the positions given.) Accordingly by shifting the double bond from $C_{6,7}$ to $C_{1,2}$ gave a linkage occurring in ergosterol and a satisfactory explanation for the observed facts. However, according to the now accepted ring system allo-cholesterol involves a shift from $C_{5,6}$ to $C_{4,5}$ giving a bonding not present in

ergosterol.



cholesterol allo-cholesterol ergosterol

Since the color reaction obtained with the trichloride and cholesterol on standing is practically the same as that color obtained when the trichloride is added to the ergosterol there is evidently some relation between the bondings and their reactions to the trichloride. From the present status, however, the shift to allo-cholesterol seems unnecessary and the time factor may be explained by the greater unsaturation in ergosterol.

As to the sensitivity of the reaction it was found that in order to obtain a characteristic color in the diluted sample, i.e. 2 cc. $CHCl_3$, with the trichloride reagent 0.0302 mgs. of ergosterol or 0.1308 mgs. of cholesterol were required. With the pentachloride reagent 0.0302 mgs. of ergosterol or 0.0654 mgs. of cholesterol were required.

Since the Rosenheim reaction is stated to be characteristic of ergosterol a comparative run was made using the Rosenheim reagent and the antimony reagents. In this

series the same amount of the sterols was used in each test. Since the sterols were dissolved in alcohol this was first removed by evaporating just to dryness on a water bath; then the sample for the Rosenheim test was dissolved in 1 cc. of $CHCl_3$ before treatment with the trichloracetic acid reagent. Under these conditions no color developed with the Rosenheim reagent and either of the two sterols. With either antimony reagent a very definite color is produced. (See Series M-1).

Since Rosenheim states that in his tests 0.01 mg. of ergosterol yields a marked color reaction within five minutes and in this case 0.1298 mg. of ergosterol was found to give no reaction it was decided to make up a fresh sample of ergosterol in $CHCl_3$ because of the possibility of some interference with the Rosenheim test on evaporating the alcohol. It was pointed out by Rosenheim that in determining the sensitivity of the reaction 0.1 cc. of the chloroform solution containing known amounts of ergosterol was taken and treated with 3 drops of the reagent. He further pointed out that the color is rapidly discharged by water or alcohol and less rapidly in $CHCl_3$. 0.5 cc. of the ergosterol in $CHCl_3$, containing 0.114 mgs. of ergosterol, was treated with 15 drops of the Rosenheim reagent. No color resulted on adding the reagent, although on standing for one hour after the addition of 15

drops more of the reagent a faint green color developed, (GY Tint #2). Using the same amount of the ergosterol solution and adding 0.22 cc. of the pentachloride reagent a brilliant orange color (O normal) developed at once. On dilution with 2 cc. $CHCl_3$ a red color (R Tint #1) resulted. On standing for one hour this color changed to violet (VR shade 1). Using the trichloride reagent and the same amount of ergosterol solution a red color (R Tint #2) developed at once. On dilution with 2 cc. $CHCl_3$ this color changed to a violet shade (RV Tint #2). On standing for about 1 hour the trichloride test became colorless. (See Series M-2.)

These results would seem to indicate that either of the antimony reagents is more sensitive for the detection and differentiation of ergosterol and cholesterol than the Rosenheim reagent.

To carry out the differential tests the following procedure is recommended.

0.5 cc. of a chloroform solution containing about 0.1 mgs. of the sterol is treated with 0.5 cc. of a saturated solution of $SbCl_3$ in $CHCl_3$. If the sterol is ergosterol a red color develops at once. On dilution to 2 cc. with $CHCl_3$ a violet shade results. With cholesterol under the same conditions no color results.

OESTRONE, OESTRADIOL, & OESTRIOL

Oestrone yielded satisfactory results in the regular test procedure with only the antimony trichloride. According to Hawk & Bergheim[15] oestrone does not give the cholesterol tests. It will be noted that oestrone has a saturated B ring, while the sterols have an unsaturated B ring. With the pentachloride reagent if any color developed it was a yellow color which has been previously discussed. With the trichloride reagent it was found possible to detect 0.004 mgs. of oestrone by comparison with a control. Regarding this limit of sensitivity, in a communication from Dr. Oliver Kamm of the Parke-Davis Co., who kindly contributed the oestrone, he pointed out that a color reaction to be of maximum value should be able to detect 5 rat units (1 rat unit equals 0.0005 mg.) These results are somewhat disappointing from this standpoint. However, as will be shown later, $SbCl_3$ can be used to give a specific color test with oestrone and oestriol the urinary hormones.

A point of difficulty in explaining the reaction is the appearance of the fluorescence. From the structure of oestrone it is impossible for the author to account for this phenomenon in the light of the accepted theory.

According to the Kaufman theory (Cohen[7]) the benzene ring is not of itself fluorescent but requires a fluoro-

gen group such as the ethylene bond occurring in phenanthrene and anthracene. Even considering the possibility of dehydration which may occur in any test that is heated there is no apparent mechanism capable of producing a fluoregen group. On heating due to the evaporation of the solvent and the fact that the melting point of the trichloride is 72°C, the action is between the fused chloride and the substance being tested. A point indicating that dehydration may have taken place is the development of a turbidity when the samples are heated. This is evidently the oxychloride since on adding HCl the turbidity is removed.

In an attempt to improve the 'fastness' of the color obtained it was found that a solution of $SbCl_3$ in concentrated HCl yielded a much better color than a solution of $SbCl_3$ in $CHCl_3$. Accordingly the three hormones were tested with this acidic solution.

Because of the similarity of the metals antimony and arsenic it was decided to determine whether the antimony reagent could be utilized as a specific test for oestriol. David's test[9] is specific for oestriol and uses arsenic acid. Accordingly the following tests were carried out. (See Series Q.) Three tubes, each containing 30 gamma, (the quantity recommended by David for his test) of the individual hormone were heated for two to three minutes

on a water bath with 0.1 cc. of concentrated H_2SO_4. When cool 0.5 cc. of water and three drops of the acid $SbCl_3$ reagent were added and the tubes then reheated on the water bath for seven minutes. Under these conditions a blue color (BG Tint #1) developed with oestriol. Oestrone and oestradiol failed to give a color reaction. By this means ten gamma of oestriol can readily be detected. The test is somewhat more sensitive than the David test which requires about 30 gamma.

As has been shown $SbCl_3$ has a dehydrating action and since in all cases a dehydrating medium is apparently necessary a series of runs were made using the $SbCl_3$ as both a dehydrating and coloring agent simultaneously. Three separate tubes, each containing about .30 gamma of one of the hormones, was heated with 0.05 cc. of the acid $SbCl_3$ reagent for 30 minutes. This time was separately determined to be the optimum for color development. When cool 0.5 cc. of water and 7 drops of concentrated HCl (to suppress hydrolysis of the $SbCl_3$) were added to each tube. Under these conditions oestrone and oestradiol yielded a red color (OR Tint #2). Oestriol failed to give a color reaction. Four gamma (8 rat units) of oestrone produces a definite color which is permanent for at least three or four hours.

TESTOSTERONE ACETATE

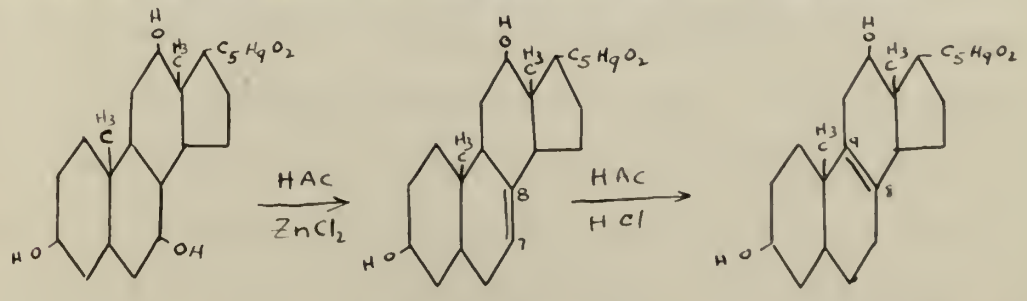
With testosterone acetate no satisfactory results were obtained using the normal procedure employed in the tests. However, it was noted that if the testosterone acetate was heated with the trichloride for about ten minutes and then allowed to stand for about one hour that a blue liquid was produced with a peculiar red fluorescence. It was felt that heating at a higher temperature might cause the color to develop more rapidly and accordingly tests were carried out (See Series L) using 0.1 cc. of testosterone acetate (corresponding to 0.075 mgs.) to which was added 0.2 cc. of the trichloride reagent and then heated for ten minutes on an oil bath at 150°C. The undiluted sample so treated showed a violet color (RV Shade 2). On diluting with 1 cc. of $CHCl_3$ the color became bluer (BV Tint #1) but was somewhat cloudy. Since this was believed to be the oxychloride it was desirable to add concentrated HCl. Past experience had shown that a single phase could only be obtained by adding alcohol, consequently 0.5 cc. of alcohol was first added. This caused a color change to blue (GB normal). On adding 0.5 cc. of concentrated HCl a clear blue solution resulted (B shade 1). A sample of oestrone treated simultaneously yielded the following results successively -- undiluted, VR normal -- on diluting with $CHCl_3$, VR Tint #2 (turbid) -- on adding alcohol, OR Tint #2 (turbid) -- on adding concen-

trated HCl, VR Tint #2 (clear). This seems to indicate the possibility of a differential color test for the male and female sex hormones using the trichloride reagents since at each step there is a pronounced color difference. This is only and indication, however, since the amount of testosterone acetate is nearly eight times that of the oestrone.

When treated with the $SbCl_3$ in HCl reagent (hormone reagent) testosterone acetate yielded a greenish-blue color with either procedure. If treated first with concentrated H_2SO_4 and then with the hormone reagent about twelve gamma could be detected. Using the hormone reagent alone about the same sensitivity was found. The tests are carried out in the same manner as that described under the female sex hormones. As one capon unit is approximately ten gamma the test may be of practical importance. A point of interest is the fact that of the four hormones tested, oestrone, oestradiol, oestriol, and testosterone acetate the latter is the only one that produces a positive color reaction with either test. It will be recalled that the sulphuric acid - hormone reagent test is specific for oestriol, while the hormone reagent test alone produces a color with oestrone and oestradiol.

BILE ACIDS

Concerning the bile acids the only samples producing other than a yellow color were cholic and apocholic acids. It was found that on standing overnight with either antimony reagent cholic acid yielded an insoluble blue precipitate (GB normal) with a clear green supernatant liquid (BG Tint #1). Since the antimony reagents are readily hydrolyzed it appeared that this color reaction might be a modification of the Hammersten[14] reaction for cholic acid. In this test powdered cholic acid when treated with 25% HCl slowly yields blue, green, and finally yellow colored solutions. The conclusion seemed more justified when it was found that apocholic acid gave the same results. Boedecker[3] prepared apocholic acid and its isomer 3,12 dihydroxycholenic acid by the use of mild dehydrating agents on cholic acid.



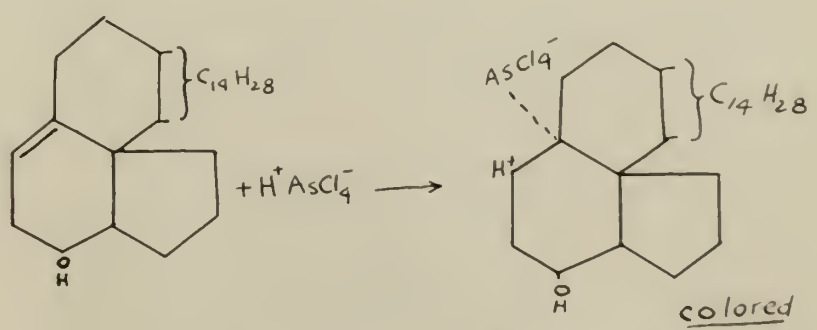
cholic acid — 3,12 dihydroxy cholenic acid — apocholic acid

Accordingly the following tests were carried out (See Series N). An equal number of mols of cholic acid and apocholic acid in alcohol were treated with sufficient HCl to give a final solution of 25%. Since the apocholic acid is

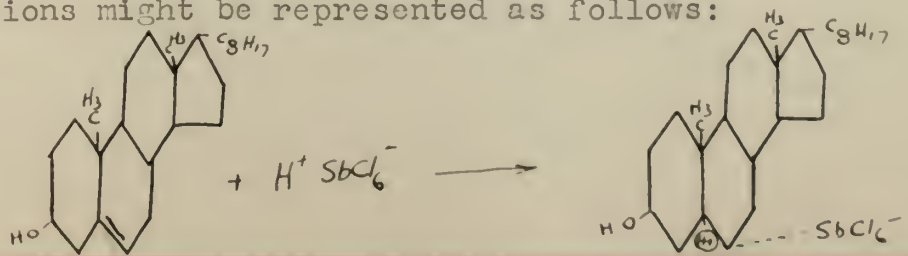
derived from cholic acid by dehydrogenation and is consequently unsaturated it should react more rapidly than its parent substance. If the color is due to the formation of a halochromic salt with the apocholic acid it should react immediately to give a color. It was found, however, that the apocholic acid required about one hour to give a yellow color (Y Tint #2). After standing for the same length of time the cholic acid showed no color. On standing overnight both samples showed the same color (Y Tint #1) with a slight green fluorescence. This is interpreted as indicating that in the Hammersten reaction the first step is one of dehydration with the introduction of an unsaturated linkage which will allow for the formation of the colored complex. With regard to the fact that the apocholic acid requires a certain period of time before reacting it is possible that the apocholic acid first undergoes isomerization to the more labile form 3,12 dihydroxycholenic acid. In this regard it should be noted that apocholic acid cannot be hydrogenated while its isomer 3,12 dihydroxy cholenic acid is readily hydrogenated to give desoxycholic acid. (Fieser, page 133). It is this labile form which couples to form the colored complex. Moreover, such a shift would yield a $C_{7,8}$ linkage such as occurs in ergosterol and would be in complete accord with the idea of Rosenheim that the sterols shift to the ergosterol linkage before reacting. Against the possibility

are the findings of Yamasaki (Fieser, page 133) that strong acids at room temperature favor the formation of the apocholic acid. It is possible, however, that under the considerations of this test the explanation offered above is valid. It will be noted that it is only apocholic acid which has an unsaturated linkage and cholic acid which readily yields an unsaturated linkage under test conditions that a distinct color is produced. The other bile acids sometimes develop a yellow color with the pentachloride on strong heating. This believed to be due to the dehydrating action of the fused chloride and the chlorinating effect as discussed under phenanthrene.

Rosenheim proposed that the formation of the colored complex could be represented in general thusly;



Modifying this idea to the present ring system the reactions might be represented as follows:



PHENANTHRENE

Phenanthrene although unsaturated produced no characteristic color complex. With the trichloride reagent no reaction was obtained even after heating for twenty minutes. With the pentachloride a yellow color was obtained only on heating. This undoubtedly the yellow tetrachlorphenanthrene which has been prepared by Zetter[51] (See page 3).

VITAMIN D_2

Vitamin D_2 is extremely reactive towards the antimony reagents. A sample of 0.0082 mgs. of this compound yields a distinct yellow color. However the color produced with the antimony reagents is not specific since Heilbron[17] (1929, page 2248) showed that an isomer of ergosterol, Ergosterol D developed a yellow color with the antimony chloride reagent.

It should be pointed out that Vitamin D_2 is an extremely labile substance and is only stable for about eight hours in $CHCl_3$. Consequently any tests carried out on this substance when dissolved in chloroform should clearly indicate the length of time that the compound has been in solution. In the tests carried out by the author the solution was made up and the tests completed within six hours.

EFFECT OF SOLVENT

The final point studied was the effect of the solvent. For these tests, with the exception of Vitamin D_2, the same number of mols of the test substances were treated with the same weight of the trichloride or the pentachloride in order that the tests might show solvent action alone. In agreement with the findings of Steinle & Kahlenberg[40] and Rosenheim[32] it was found that the complex was unstable in alcohol for either of the antimony reagents even when HCl was added as a stabilizer. With the pentachloride in glacial acetic acid only oestrone, cholesterol, and ergosterol gave stable colored complexes. These complexes were respectively green, (G broken tone), blue-green (BG broken tone), and blue-violet (BV shade 1). The trichloride in glacial acetic acid produced no color with any of the substances tested. Preliminary tests on the effect of the solvent were made using ether, methyl alcohol, n-propyl alcohol, n-butyl alcohol, sec-butyl alcohol, ethylene glycol, glycerol, and carbon tetrachloride. These solvents were found to be unsatisfactory either because the compounds themselves were insoluble or because the solvent produced a fading action resulting in a yellow color.

With regard to the effect of the solvent on Vitamin D_2 the results were substantially the same as with the other compounds tested although the pentachloride in alcohol did

develop a faint color (GY tint #2).

SUMMARY

The results indicate, as has been shown by previous workers, that the most suitable organic solvent for the test reagents is chloroform.

The tests show that the antimony chlorides are sensitive reagents for the detection of the sterols and consequently require a high degree of purity in the sterols being tested if the colors obtained are to be consistent. Of particular significance is the ability of the antimony chlorides to differentiate between ergosterol and cholesterol even more satisfactorily than the Rosenheim test.

With the exception of cholic and apocholic acids normal test conditions yield no significant color with the bile acids. The test obtained with these two named bile acids is undoubtedly a modification of the Hammersten test.

A limitation on the use of the pentachloride as a test reagent is its chlorinating action when heated. The trichloride, on the other hand, may only produce colors when the samples are heated. This is believed to be due to its dehydrating action.

A procedure is outlined which would differentiate between the urinary hormones oestrone and oestriol using the antimony trichloride in concentrated HCl. The test is sensitive to about 4 gamma for the oestrone and 10 gamma for the oestriol.

With antimony trichloride in concentrated HCl testos-

terone acetate develops a definite color in either of the procedures used to differentiate the female sex hormones. The test with this hormone is sensitive to about 12 gamma.

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V I T A

John Kiernan Rouleau

Born December 13, 1906 in Boston, Massachusetts.

The son of Albert Joseph Rouleau and

Margaret (nee Kiernan) Rouleau

Attended local grade schools in Boston and

Entered St. John's Preparatory School, Danvers, Mass. in 1919 and graduated in 1923.

Entered Massachusetts Institute of Technology in 1924 and graduated with the degree of Bachelor of Science in Chemical Engineering in 1928.

From 1928 to 1930 employed as chemist for the Vadsco Sales Corporation in New York, N.Y.

From 1930 to 1932 employed as Instructor at Massachusetts Institute of Technology in the Department of Chemical Engineering.

Degree of Master of Science in Chemical Engineering received from Massachusetts Institute of Technology in 1932.

Engaged by the State of Massachusetts as chemical assistant in the Department of Public Health, in 1932.

Employed by the Merrimac Chemical Co. as research engineer until May of 1933.

Appointed Teaching Fellow in Bio-chemistry at Boston University School of Medicine in 1933 until May of 1935.

During this period took courses at Boston University and Medical School.

Employed as Instructor in the Chemistry Department of Boston College from October 1935 to date.

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1 1719 02556 8512